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Classification of Cannabis Plants Grown in Northern Thailand using Physico-Chemical Properties

Prapatsorn Tipparat[1*], Weerapun Kunkaew[2], Suthat Julsrigival[2]
Sarita Pinmanee[3], Surapol Natakankitkul[4]
1 Regional Medical Sciences Center 1, Department of Medical Sciences, Chiang Mai 50180, Thailand.
2 Royal Project Foundation, Chiang Mai 50200, Thailand.
3 Highland Research and Development Institute (Public Organization), Chiang Mai 50200, Thailand.
4 Faculty of Pharmacy, Chiang Mai University, Chiang Mai 50200, Thailand.
* E-mail of the corresponding author: ptipparat@yahoo.com

Abstract
The Thai government has recognized that hemp may make useful contributions to the economy as an alternative crop. This study was conducted to provide information in both chemical and physical characters of Thai-grown cannabis for breeders to discriminate their phenotypes and accessions in order to select the low intoxicant with high fiber producing cultivars. The cannabinoids on the basis of THC, CBD and CBN content of 750 plants from eight accessions derived from five local cannabis cultivars were analyzed individually and their morphological features were also determined. According to the individual plants belonging to the same accessions showing distinct THC/CBD ratios were classified into different phenotypes, it is impossible to classify only single plant for defining the phenotype or determine cannabinoids content on the single analysis. THC content is found to correlate negatively to their physical characters such as plant height, stem diameter and fiber weight. Principal component analysis showed that the fiber weight, cork weight and stem diameter of the plant as well as chemical features such a THC content, CBD content, THC/CBD ratio and log10(THC/CBD ratio) explained most of the total variation which could distinguish accession and phenotype of the cannabis plants. Stepwise discriminant analysis confirmed that cannabinoids and some physical properties could be used to classify the phenotype of cannabis plants into drug, intermediate and fiber types as well, whereas the accessions of cannabis could not be discriminated clearly by using only their physico-chemical parameters. The genetic characteristics which affect the chemical patterns and morphological traits among cannabis accessions grown in northern Thailand should be considerable to investigate in further study.
Keywords: classification, cannabis plant**,** northern Thailand, physico-chemical properties**,** discriminant analysis, principal component analysis

1. Introduction

Cannabis is an annual plant belonging to the family *Cannabaceae* and genus *Cannabis* which has been used as psychoactive drug and as a source of fiber and seed for centuries. It is normally dioecious having male and female on separate plants, each with distinguished growth characteristics (Small & Marcus, 2002; Clarke &Watson, 2007). Cannabis contains a unique class of chemicals called cannabinoids which are a group of terpenophenolic compounds secreted as a resin component by glandular trichomes found mostly in flowering tops and bracts. Cannabinoids are produced biosynthetically as their carboxylic acid derivatives which are decarboxylated into their neutral forms through the action of heat, sunlight and storage. Δ^9-tetrahydrocannabinol (THC) is the main psychoactive constituent; other major constituents are cannabidiol (CBD), cannabichromene (CBC) and cannabinol (CBN); the degradation product of THC which is found a few in fresh plants (Clarke &Watson, 2007; Pate, 1994; Fellermeier *et al*., 2001; Flores-Sanchez & Verpoorte, 2008; Taura *et al*., 2007). The cannabinoids contents in cannabis plants are highly variable due to environmental conditions of cultivation, harvest time, storage conditions as well as genetic factors. The production of cannabinoids also increases in plants under stress condition such as weather (Latta & Eaton, 1975; Turner *et al*., 1982; Baker *et al*., 1982; Baker *et al*., 1983; Taylor *et al*., 1985; Tipparat *et al*., 2012). The cannabis species discriminated by their genetic, morphological and chemotaxanomic variation was recognized (Hillig, 2005; Hillig & Mahlberg, 2004; Hillig, 2004). However, it is still a matter of debate. As of 2007, most taxonomists have listed cannabis as a single species; *Cannabis sativa* L. (Clarke &Watson, 2007). By THC content itself, THC/CBD ratio or [THC+CBN]/CBD ratio defined as phenotypic index (Hillig & Mahlberg, 2004; Fetterman *et al.*, 1971; Small & Beckstead, 1973) has been used for classifying drug-, intermediate- and fiber phenotypes in cannabis plants. The chemotypes do not change in plants at different ages or in different sexes, as demonstrated by the consistency of major cannabinoids ratios; CBD/THC or CBG/CBD throughout the entire life cycle until flowering (Pacifico *et al.*, 2008). Several molecular techniques have also been used for distinguishing fiber-type from drug-type cannabis plants such as PCR marker for THCA synthase gene (Kojoma *et al.,* 2006), genomic DNA markers using random amplified polymorphic DNA (RAPD) (Jagadish *et al.*, 1996; de Meijer *et al.,* 2003), sequence characterized amplified region (SCAR) markers (Pacifico *et al.*, 2006) and short tandem repeats (STRs) which is

most widely used for DNA typing (Mendoza *et al.*, 2009).

Recently, more than 30 countries all over the world have grown hemp or fiber-type cannabis as potential important crop for fiber and seed production under specific permission. The THC content in industrial hemp produced from certified seed is limited to 0.2 % in European Union, 0.3 % in Canada, whereas in Queensland, Australia it has recently been raised to a level of 0.5% and up to 1 % for circumstances with elevated THC concentrations as a result of climatic or environmental changes (Mignoni, 1997; West, 1998; Department of Primary Industries and Fisheries, 2013).

In Thailand, either drug cannabis or hemp cultivation was forbidden since 1979. However, the Thai government has recognized the possibility of legitimate cultivation of hemp. The studies of cannabinoids characteristics and factors affecting their contents of Thai-grown cannabis: the cannabis landraces for fiber usage collected from local farms and the authorized cannabis cultivars grown in different trial fields, were then conducted in 2007 to establish the criteria for the regulation of cannabis cultivation in Thailand (Tipparat *et al*., 2012, Tipparat *et al*., 2009). Actually, hemp cultivation has a very long tradition among the hill tribes living in Northern Thailand. Thus, the cannabis cultivars which named according to theirs geographic origin used in the prior and following studies were derived from those local farms. The resulted indicated that most Thai cannabis landraces and in trial fields defined as intermediate type by using $\log_{10}$ (THC/CBD) values, unless their actual THC contents were higher than 1%. The researchers in a breeding program have also attempted since then to select low content of THC cultivars. The other characters such as a plant height and fiber content which are benefit for fiber production have also been interested in. Although, the relationship between chemical and other visible plant characters which would allow an indirect recognition of accessions or cultivars was reported as quite limited (de Meijer *et al.,* 1992), the information is still necessary for breeding researchers in Thailand. The study was then conducted to establish the parameters that could be discriminated Thai- grown cannabis. The cannabinoids on the basis of THC, CBD and CBN content and physical properties were therefore investigated.

2. Materials and methods

2.1 Plant materials

Seven hundred fifty cannabis samples collected from a trial field authorized by the Food and Drug Administration of Thailand were analyzed. The experiment was conducted during June-December, 2009 at Pang Da Royal Agricultural Station, the highland growing area of the Royal Project Foundation in Samoeng District, Chiang Mai Province, Thailand; with an average elevation of 720 m. ASL, average temperature of 18.8-29.6 °C, 1,075 mm rainfall and average relative humidity of 53.6-95%. Eight seed stocks of five local cannabis cultivars derived from different ecological areas in the previous year were collected and cultivated (see Table 1). Each of them was grown in 10 m. X 10 m. trial field with plastic shelter. All cultivated areas were treated in the same conditions with distance between rows of 75 cm, between pots of 25 cm and sowing rate of 3-4 plants per pot. No pesticides were supplied to these crops.

Individually, male and female plants of each accession were analyzed in both chemical and physical properties.

2.2 Cannabinoids analysis by GC-FID

The leaves randomly collected from one-third of the upper part of the plant stem at early stage of flowering were dried at 40°C for 48 hours to a residual humidity of less than 10%, grounded and stored in light protection plastic bag refrigerated at 5°C until cannabinoids analysis.

The standard of delta-9-tetrahydrocannabinol (THC) cannabidiol (CBD) and cannabinol (CBN) were imported from Switzerland (Lipomed®). Each standard was diluted to 0.2 mg/ml with methanol (Merck®, USA) and 0.2 mg/ml 2,2,2 triphenylacetophenone (Merck®, USA) as internal standard was added with ratio 1:1. 500 mg of each sample was extracted with 10 ml methanol by shaking for 1 hr. The discarded extract was centrifuged at 4,000 rpm for 5 min and then 500 µl of supernatant was diluted to 1:1 with 0.2 mg/ml 2,2,2 triphenylacetophenone as internal standard. 1.0- µl aliquot of the extract was injected and quantitatively analyzed by using Chrompack® 9002 GC-FID on DB-1 capillary column (30 m x 0.32 mm I.D. film thickness 0.25) with following conditions: nitrogen as carrier gas with flow rate 2 ml min^{-1}; split ratio 1:20; injector and detector temperature, 260 and 270°C respectively; oven temperature programmed, 7 min. at 230 °C, increase to 260 °C at 10 °C/min, hold at 260 °C for 2 min (Tipparat *et al*., 2012). Most of cannabinoids in fresh plant materials exist in the form of their acids precursors, however, the high temperature that is applied in GC causes the decarboxylation of acidic to their corresponding neutral forms (Hazekamp *et al*., 2005). THC CBD and CBN contents were thus quantified upon the peak area ratio of each cannabinoids to internal standard in sample comparing with the peak area ratio of each standard to internal standard.

2.3 Physical properties analysis

At inflorescence stages, male plants flower earlier than female plants so that their appearance can be distinguished. Seven physical features were determined individually in both male and female plants as follows: plant height (m) and number of branch measured from soil level to the last stem node, stem diameter (mm) and

internode length (cm) measured at the height of 1 meter from soil level, whereas cork weight (g), fiber weight (g) and fiber content (%) measured on dry weight of 40 cm of stem at the height of 1 meter from ground level after harvesting.

2.4 Statistical Analysis

Data were interpreted by the univariate and multivariate statistical analyses by using the SPSS software version 17.0. The significance differences between means were detected by the Duncan's multiple range test ($p<0.05$). The chemical and physical variables were standardized prior to the analysis. Principal component analysis with varimax rotation method was applied to determine the important factors for further classifying. Discriminant analysis was performed according to the variables extracted from previous factor analysis in order to verify the prior phenotypes classified by using $\log_{10}$ (THC/CBD) values (Table 1) and the accessions of cannabis defined from their origin seed stocks.

3. Results and discussion

3.1 Chemical and physical features

The 750 cannabis samples from 8 accessions were analyzed for chemical and physical characters as shown in Table 2. According to cannabinoids contents did not change in leaf samples even dried at 35°C for 1 week and stored at a temperature of 40°C during 6 weeks in the dark (de Meijer *et al*., 1992) and CBN was also not present in fresh samples of cannabis (Turner *et al*., 1982), the chemical features in this study were thus established only on the basis of a plant's dry weight of THC and CBD; the major cannabinoids but ignoring CBN by using the verified GC method following Tipparat *et al*., 2012. A scatter plot between THC and CBD content of each group in Fig.1 illustrated that individual plants belonging to the same populations showing distinct THC/CBD ratios were classified into different phenotypes, which had altered patterns among accessions. Cannabis plants from seeds representing the same variant but different origins such as; HPD-M1-VN and HM1-VN; HPD-M1-MM and HM1-MM tending to have similar distributions, whereas HPD-M1-PU and HM1-PU which were different, suggested the existence of genetic variation. Mandolino *et al.*, 2003 and de Meijer *et al*., 2003 reported that phenotypes or chemotypes could be expressed as THC/CBD ratio and appeared to be under the genetic control of one single locus endowed with two co-dominant alleles which elucidated the tripartite distribution of the chemotypes within populations. Although, the use of molecular markers is able to determine phenotype at early stage that may be useful for breeding program, it cannot provide any information on the amount of cannabinoids produced by the plants. A histogram shown in Fig.2 explicated the different characteristic of THC distribution. The results indicated that it is impossible to classify only single plant for defining the phenotype or determine cannabinoids content on the single analysis. For regulation, the sample used is necessary to be representative and big enough to assure the average concentration of the constituent as the same of population. It could calculate the minimum number of sampling plants from data on the variability of the constituent following a normal Gaussian distribution (Mechtler K. *et al.,* 2004). By the results of this study, a sample size of 50 plants should be considerable for routine analysis as mentioned by EU regulations. Unless the results are not clear, bigger samples are collected.

As shown in Table 2, univariate statistics and significant differences of cannabinoids content and physical characters between the plants cultivated from seed stocks which grown in different ecological areas in previous year were illustrated. The mean actual THC and CBD contents of each accession were quite fluctuating due to genetic characteristics of seed stocks, whereas mean THC/CBD ratio of the same variant such as VN, MM and PU inclined to consistency but having the lower values than theirs parental. However, they did not differ significantly from the other accessions. Nevertheless, the physical appearances were high deviation among accessions.

3.2 Relationship between chemical and physical features

The Pearson correlation coefficients as presented in Table 3 illustrated the association between chemical and physical features. The chemical characters such as THC content along with log10 (THC/CBD ratio) trended to correlate negatively to their physical characters. Among the physical parameters, the plant height showed positive correlation significantly to number of branch, internode length, stem diameter, cork weight and fiber weight, consequently. Especially, stem diameter and cork weight of the plants affected strongly to their fiber weight as revealed by de Meijer, 2004.

3.3 Multivariate patterns of cannabis accessions and phenotypes

Therefore, principle component analysis (PCA) with varimax rotation method was applied to classify cannabis accessions and theirs phenotype as pre-defined following the criteria as mentioned in Table 1. Chemical and physical variables were grouped into four components explaining 77.1% of the total variance as shown in Table 4. The highest explain variance of 32.4% for PC1 was associated with fiber weight, cork weight and stem diameter of the plant, respectively. The second factor (PC2) explained 19.9% of variance involving with chemical features such a THC content, CBD content, THC/CBD ratio and log10(THC/CBD ratio). Whereas, PC3 which explained 14.6% of variance related with plant growth variables for instance; plant height, number of

branches and their internode length. The last factor (PC4) explained for fiber content with 10.1% of variance. From scatter plot of PC1 versus PC2 and PC1 versus PC3 of all cannabis plants illustrated in Fig.3, HPD-M1-PU, HM1-PU and HM0-DP accessions which had high level of THC but low level of CBD could be separated along the positive PC2, while HPD-M1-VN and HM1-VN; the low level of THC but high level of CBD accessions, were separated along the negative PC2. Meanwhile, the medium THC accession such as HPD-M1-MM and HPD-M1-HH were not well distinct. According to the cannabinoids content and theirs ratio, three phenotypes were also distinguished clearly. Along PC3 axis, it however could not discriminate well in both accessions and their phenotypes.

Stepwise discriminant analysis was used to confirm the PCA analysis and to predict the accessions and phenotypes of cannabis plants. Predictor variables for accessions were THC content, CBD content, log10 (THC/CBD ratio), plant height, stem diameter, number of branches, internode length, cork weight and fiber content. The discriminate function revealed a significant association between accessions and all predictors accounting for 66.58% of between accession variability with some predictors such as plant height, internode length, their number of branches, log10 (THC/CBD ratio) and CBD content that indicated the largest correlation. The cross validated classification showed that only overall 54.5% were correctly accession classified. HM1-PU was classified with slightly better accuracy (92.3%) than HPD-M1-PU (70.3%), HPD-M1-VN (58.0%) and HM1-VN (56.9%), respectively. Meanwhile, their phenotypes were well discriminated resulting in 98.3% correct assignment of the plants to drug, intermediate and fiber types. The fiber types were distinguished with the best accuracy (100.0%) following by intermediate (98.4%) and drug type (88.6%). The variables used in their classification function were THC content, THC/CBD ratio, log10 (THC/CBD ratio), stem diameter and accession of the plants with a canonical correlation of .825 explaining 68.06% of the variation. Scatterplot of 750 Cannabis plants on the 1st and 2nd canonical discriminant functions elucidating the classification of accessions and phenotypes of the cannabis plants illustrated in Fig. 4.

4. Conclusion

As a result, variation of chemical features due to the distinct of THC and CBD content as well as physical appearances of the individual plant within accessions is observed. Although, the mean actual THC and CBD contents of each accession are fluctuating, the means THC/CBD ratio of those belonging to the same variant are consistency. According to the variability of THC values in one population, the minimum number of 50 plants is considerable as representative to be sampled for analysis. Nevertheless, THC content is found to correlate negatively to their physical characters such as plant height, stem diameter and fiber weight. This association will be helpful for a breeding program. From factor analysis and discriminant analysis, it could be confirmed that the chemical and some physical properties could be used to classify the phenotype of cannabis plants grown in northern Thailand into drug, intermediate and fiber types as well. Therefore, the log10 (THC/CBD ratio) parameter is preferable to use for distinguishing the phenotype of cannabis. However, the accessions of cannabis could not be discriminated clearly by using only their physico-chemical parameters conducted in this study due to the variation on morphology and chemical composition of the plants which were influenced from not only genetic characteristics of seed-stocks but also environmental factors presented in their parental growing areas such as climates and elevation of cultivated area. The genetic relationships among cannabis accessions grown in northern Thailand will be performed in further study.

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Table 1 Criteria for the classification of phenotypes according to phenotypic index defined by Hillig & Mahlberg, 2004

Phenotype	**log10 (THC/CBD)**	**THC/CBD**
drug type	>1	>10
intermediate type	between -0.7 and 1	Between 0.2 and 10
fiber type	<-0.7	<0.2

Table 2 Chemical and physical features of cannabis plants grown in 2009 at Pang Da Royal Agricultural Station

accessions	Plants sample (n)	THC (% d.w.)	CBD (%d.w.)	THC/CBD ratio	Log10 (THC/CBD ratio)	Plant height (m)	Stem diameter (mm)	NO. of branch	Internode length (cm)	Cork weight (g)	Fiber weight (g)	Fiber content (% d.w.)
		mean±SD	mean±SD	mean±SD	mean±SD	mean±SD	mean±SD	mean±SD	mean±SD	mean±SD	mean±SD	mean±SD
HPD-M1-VN	138	0.214±0.22^{a}	0.982±0.43^{d}	0.68±4.67^{a}	-0.88±0.66^{a}	3.92±0.38^{d}	20.89±4.78^{c}	21±4de	18.96±3.40^{d}	24.42±13.45^{de}	3.15±1.53^{bc}	11.98±2.71^{a}
HPD-M1-MM	148	0.405±0.34^{b}	0.633±0.42^{c}	1.81±4.03^{ab}	-0.19±0.60^{c}	4.02±0.39^{de}	20.84±5.32^{c}	22±4^{e}	22.54±4.07^{f}	21.23±10.94^{cd}	3.39±2.32^{cd}	13.78±4.02^{bcd}
HPD-M1-HH	129	0.396±0.35^{b}	0.546±0.40^{bc}	1.73±2.62^{ab}	-0.22±0.70^{c}	4.10±0.32^{e}	22.81±5.12^{d}	22±5^{e}	20.28±3.61^{e}	26.60±14.76^{e}	4.06±2.15^{de}	13.62±2.46^{bc}
HPD-M1-PU	145	0.552±0.40^{c}	0.344±0.37^{a}	5.18±7.03^{c}	0.36±0.59^{d}	3.94±0.34^{d}	18.64±4.59^{b}	18±4^{c}	19.17±2.83^{d}	18.66±12.60^{bc}	2.91±1.80^{bc}	14.05±2.35^{cd}
HM1-VN	51	0.454±0.39^{bc}	1.307±0.62^{e}	0.56±0.91^{a}	-0.59±0.56^{b}	3.03±0.52^{b}	17.10±5.18^{b}	15±4^{b}	14.99±3.15^{b}	14.86±10.04b	2.53±1.58^{b}	15.13±3.02^{e}
HM1-MM	49	0.883±0.65^{e}	0.945±0.74^{d}	1.74±1.87^{ab}	-0.03±0.52^{c}	3.49±0.44^{c}	21.99±6.40^{cd}	17±3^{c}	16.71±2.90^{c}	26.62±19.87^{e}	4.36±2.74^{e}	14.74±2.02^{de}
HM1-PU	40	1.209±0.65^{f}	0.312±0.20^{a}	4.33±1.90^{bc}	0.60±0.17^{e}	2.48±0.39^{a}	11.55±3.06^{a}	11±3^{a}	11.30±1.91^{a}	8.48±4.03^{a}	1.50±0.69^{a}	15.54±2.65^{e}
HM0-DP	50	0.684±0.41^{d}	0.439±0.47^{ab}	8.24±28.68^{c}	0.33±0.62^{d}	3.57±0.21^{c}	25.23±6.54^{e}	21±3^{d}	15.53±2.64^{b}	36.29±20.94^{f}	5.39±3.31^{f}	12.87±2.39^{ab}

Note: The seed stocks from the previous year which grown at different ecological areas in Northern Thailand were used in this experiment:
HPD-M1-VN, HPD-M1-MM, HPD-M1-HH and HPD-M1-PU: VN, MM, HH and PU cultivars grown at Pang Da Royal Agricultural Station with an average elevation of 720 m. ASL, average temperature of 18-29 °C, 1,075 mm rainfall and 54-95% average RH
HM1-VN: VN cultivar grown at Ang Khang Royal Agricultural Station with an average elevation of 1,400 m. ASL, average temperature of 19.5 °C, 1,937 mm rainfall and 74.6% average RH
HM1-MM: MM cultivar grown at Mae Sa Mai Agricultural Station with an average elevation of 900 m. ASL, average temperature of 19-28 °C, 1,270 mm rainfall and 88% average RH
HM1-PU: PU cultivar grown at Pang Ung Agricultural Station with an average elevation of 1,240 m. ASL, average temperature of 14-22 °C, 1,995mm rainfall and 70.1% average RH
HM0-DP: DP cultivar grown in landrace at Doi Pui with an average elevation of 1,685 m. ASL, average temperature of 2-23 °C, 1,350-2,500 mm rainfall and 70-80% average RH
Superscripts with the same letters in column do not differ significantly by DMRT ($p<0.05$)

Table 3 Correlation of chemical and physical features of cannabis plants

	THC content	CBD content	THC/CBD ratio	log10 (THC/CBD ratio)	plant height (m.)	stem diameter (mm)	No. of branch	internode length (cm)	cork weight (g)	fiber weight (g)	fiber content (%)
THC content	1										
CBD content	-.208**	1									
THC/CBD ratio	.225**	-.280**	1								
log10(THC/CBD ratio)	.637**	-.670**	.465**	1							
plant height(m.)	-.290**	-.112**	-0.028	-.096**	1						
stem diameter(mm)	-.162**	0.047	-0.03	-.118**	.451**	1					
No. of branch	-.269**	-0.024	-0.041	-.164**	.666**	.489**	1				
internode length (cm)	-.213**	-0.038	-.081*	-.086*	.560**	.149**	.244**	1			
cork weight (g)	-.132**	0.055	-0.02	-.115**	.295**	.894**	.415**	-0.021	1		
fiber weight (g)	-.098**	0.05	-0.018	-0.059	.281**	.824**	.327**	0.031	.875**	1	
fiber content (%)	.122**	-0.002	0.016	.163**	-.168**	-.248**	-.298**	0.012	-.307**	.118**	1

** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

Table 4 Factor loadings of rotated chemical and physical features of cannabis plants

		Principle component			
		Factor 1	**Factor 2**	**Factor 3**	**Factor 4**
	Variation explained (%)	32.41	19.93	14.63	10.15
Principle component loadings	THC content		**0.630**		
	CBD content		**-0.774**		-0.246
	THC/CBD ratio		**0.635**		
	log10(THC/CBD ratio)		**0.930**		
	plant height(m.)	0.273		**0.859**	0.278
	stem diameter(mm)	**0.912**			-0.387
	No. of branch	0.389		**0.592**	
	internode length (cm)			**0.828**	
	cork weight (g)	**0.950**			
	fiber weight (g)	**0.958**			
	fiber content (%)				**0.948**

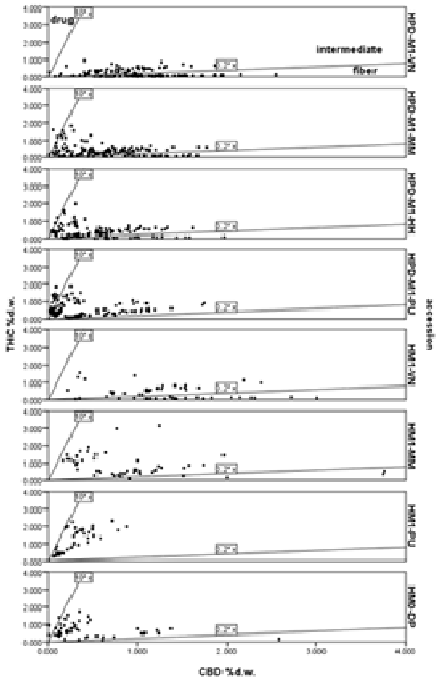

Fig. 1 Plot of THC versus CBD content of individual plants belonging to eight populations; the criteria defining into three phenotypes following Hillig & Mahlberg, 2004

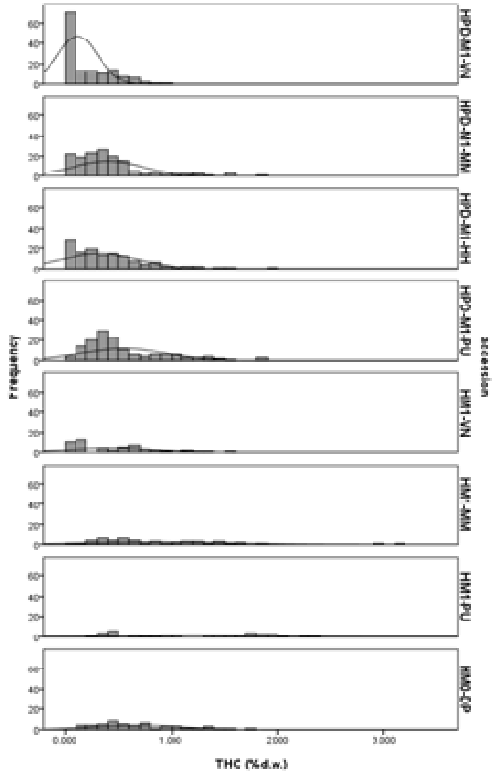

Fig. 2 THC histogram; frequency of individual plants versus THC content (%d.w.)

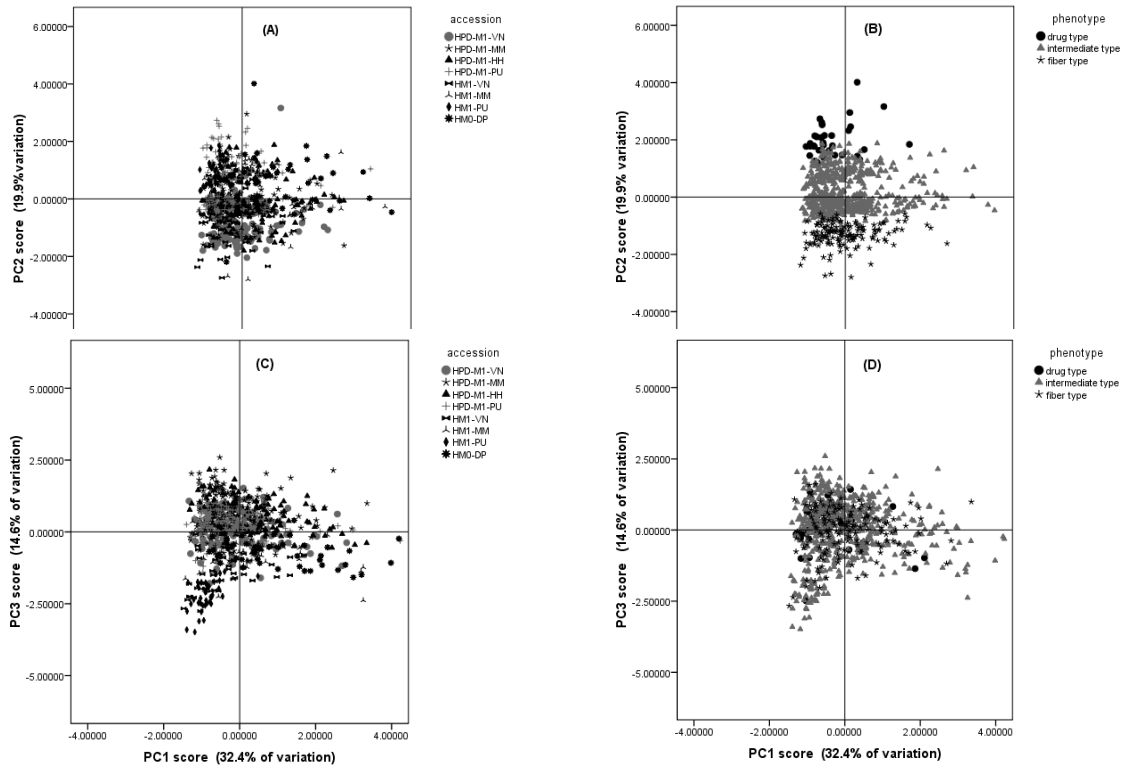

Fig.3 PCA of 750 cannabis plants; Scatter plot of PC1 versus PC2 and PC1 versus PC3 comparing separation of accessions (A,C) and phenotypes (B,D)

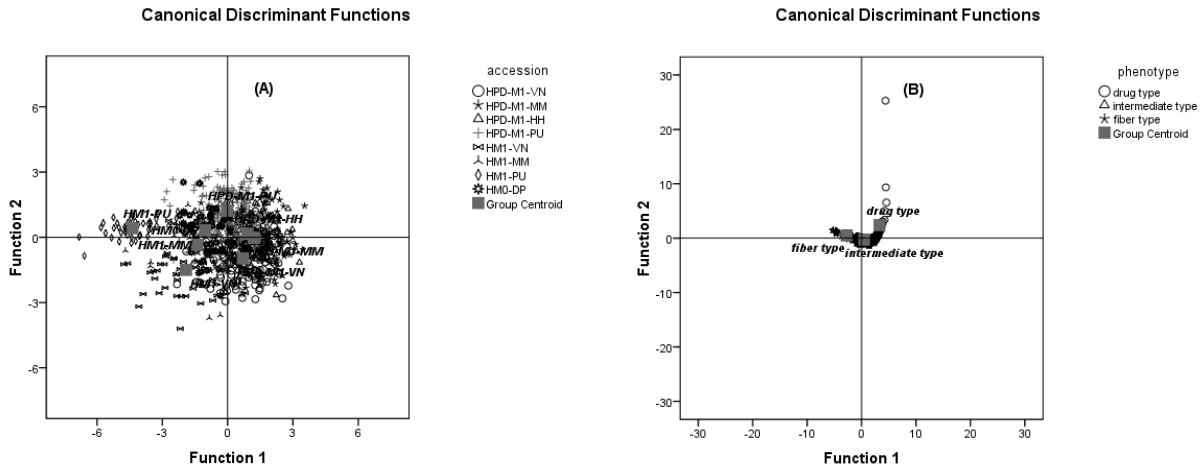

Fig.4 Scatterplot of 750 Cannabis plants on the 1st and 2nd canonical discriminant functions illustrated the separation of accessions (A) and phenotypes (B)