Biochemical differences in *Cannabis sativa* L. depending on sexual phenotype

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Abstract. Hemp (*Cannabis sativa* L.) is a species considered as having one of the most complicated mechanisms of sex determination. Peroxidase and esterase isoenzymes in leaves of the two sexual phenotypes of hemp were studied. Significant differences in isoperoxidase and isoesterase patterns were found between male and female plants, both in the number and stain intensity of bands. For both esterase and peroxidase, the isoenzymatic spectrum is richer for staminate plants. Also, some differences are obvious between the two sexes concerning catalase and peroxidase activities, as well as the level of soluble protein. The quantitative analysis of flavones, polyholozides and polyphenols emphasized differences depending not only on sex, but also on tested organ.

Key words: electrofocusing, hemp, isoenzymatic pattern, secondary metabolites, sexual phenotype.

Introduction

The chemical composition of hemp (*Cannabis sativa* L.) is very complex, including about a hundred of compounds isolated from hemp organs: flavonoids, fatty acids, phenolic spiroindans, dihydrostilbenes, nitrate substances (amines, ammonium salts, spermidine-derived alkaloids), etc. The hemp flavour is due to volatile terpenic compounds of essential oils, monoterpenes representing 47.9-92.1% and sesquiterpenes 52-48.6% of total terpenes. Compounds like friedeline, epifriedelinol, β-sitosterol, carvone and dihydrocarvone were isolated from roots (SETHI et al. 1978). Seeds contain oils (PETRI 1988), while among plant organs

flowers are richer in oils than leaves (LEMBERKOVICS et al. 1979). The fatty acid composition of fruits is of great interest, because of their use for nutritive and pharmaceutical purposes. If the complete fruit and seed are similar in this aspect, some differences are in the outer layer (MÖLLEKEN, THEIMER 1997). Although we have not found any systematic study on flavonoid synthesis in the genus *Cannabis* there are a few papers regarding these compounds in hemp (BATE-SMITH 1962, PARIS et al. 1975, 1976, PARIS, PARIS 1976, SEGELMAN et al. 1978), but the findings are contradictory, having a limited systematic value, because of the use of different analytic methods or of different plant organs or of various provenances.

The hemp-specific substances, cannabinoids, include more than 70 substances, such as Δ^9-THC (tetrahydrocannabinol), CBD (cannabidiol) and CBN (cannabinol), which are the criteria distinguishing between the hemp chemotypes (especially Δ^9-THC and CBD and THC/CBD ratio).

Although the hemp is a dioecious species, as a consequence of intensive improvement, a lot of sexual phenotypes are cultivated, the most frequent being the monoecious forms, classified in more categories, on a five-point scale, depending on female flowers/male flowers ratio. *Cannabis sativa* L. has a very complex genetic constitution and heredity, which explains the dioicism, amplitude of phenotypical variability, polymorphism and the great biological plasticity of this species. WESTERGAARD (1958) considered the sex inheritance in hemp as being one of the most complicated mechanisms among all dioecious plants. For hemp we could not find any consistent study on differentiation between sexual phenotypes, regarding morphological, physiological or biochemical traits, in spite of some disparate data. It is known that, by specific reactions, it becomes possible to make the distinction between male and female individuals of *Populus*, as well as between the – and + *Mucor* hyphae (SINNOTT 1960). Certain differences also exist between staminate and pistillate plants of *Lychnis dioica* (STANFIELD 1944, cited in SINNOTT 1960). For genera *Cannabis* and *Spinacia*, variable levels of cellular extract pH are cited, depending on sex (CHEUVART 1954). In other plant species, the analyses evidenced different values of oxidase activities in female and male individuals (AITCHINSON 1953, cited in SINNOTT 1960).

For these reasons, the principal objective of this study was to identify the existence of some biochemical differences (enzymes, secondary metabolites) in hemp, depending on sexual phenotype.

Material and methods

The studied material was collected from female and male plants of hemp (*Cannabis sativa* L. subsp. *sativa* var. *sativa*), randomly chosen from a population grown in the experimental field of the Botanical Garden of University "Al. I. Cuza" Iaşi. The seeds belonging to a fiber hemp cultivar were provided by the Agricultural

Research Centre of Secuieni at Neamţ. To estimate *in vivo* catalase and peroxidase activities, as well as soluble proteins, leaves of female and male hemp plants were used. These determinations were carried out individually, in leaves collected from 20 females and 15 males of the same age (20 weeks old). Because of the lack of simultaneity in maturity and flowering, specific for this species, the plants were in different developmental stages. The females were in the early fruit formation phase and the male plants were in full bloom.

To obtain the crude extract for the determination of the enzymatic activities and the amount of soluble protein, known quantities of well ground plant material (fine powder) in 0.01 M sodium phosphate (pH 7) were homogenized. The homogenate was maintained at $4^{o}C$ for 4 hours, and then it was centrifuged at 22 0000 rpm for 10 minutes. The supernatant was used as extract.

Catalase activity was determined by the iodometric method (ARTENIE, TĂNASE 1981). The principle of this method is based on potassium iodide oxidation by undecomposed hydrogen peroxide, after an incubation interval with catalase, followed by titration of delivered iodine with sodium thiosulfate, in the presence of starch solution as an indicator. The mixture: 0.01 M phosphate buffer pH 7, enzymatic extract and 3% hydrogen peroxide was incubated for 5 minutes. The reaction was blocked with 10% sulfuric acid. Then 10% potassium iodide and 1% ammonium molybdate were added. Titration was made with 0.1 sodium thiosulfate. The catalase activity was calculated knowing that one catalase unit is equivalent to the amount of enzyme which decomposes 1 µmol (0.034 mg) H_2O_2 during 1 minute. The results are expressed in mg H_2O_2/g fresh matter.

Peroxidase activity was quantified by the photometric method, based on benzidine oxidation under the peroxidase activity, in the presence of H_2O_2/time unit. The mixture, composed of 1% benzidine in glacial acetic acid, 3% hydrogen peroxide, and the enzymatic extract, was incubated for 3 minutes. 30% NaOH was used to stop the reaction. Absolute ethanol was added. The values of extinctions were determined with a SPEKOL 20 spectrophotometer, at $\lambda = 470$ nm. After the estimation of the ratio: sample extinction/control extinction, the peroxidase activity was expressed in mg H_2O_2/g fresh matter.

The specific activities for catalase and peroxidase were estimated by reporting the quantity of substratum consumed by enzyme to the concentration of soluble protein in 1 g of tissue. They were expressed in mg H_2O_2/mg protein.

The obtaining of enzymatic extracts used in electrofocusing involved very fine grinding of the plant material with a Potter homogenizer. The homogenate (1 : 3, w/v, in 0.1 M Tris/HCl buffer pH 7.2) was centrifuged at 17 000 rpm, with a refrigerated JANETZKI K-24 centrifuge. The supernatant was used to identify the izoenzymatic patterns. To assess the isoenzymatic pattern for esterase and peroxidase, we used electrofocusing on polyacrylamidic gel containing urea, H_2O, acrylamide, ampholine, and ammonium persulfate, according to the Wrigley method (TÓTH 1992). Ampholine pH 3.5-10 (LKB) was used to establish the pH gradient. The pH was controlled with a digital RADELKIS 20 pH meter. The sep-

aration was achieved in the glass test tubes 0.5×7.5 cm of the electrophoretic apparatus, in a disk of SHANDON type. The peroxidase visualization was performed with o-dianisidine (MCDONALD, SMITH 1972), at pH 4.8, established with 0.2 M sodium acetate buffer. The esterase visualization was conducted according to SCANDALIOS (1969). In the solution for incubation (0.15 M phosphate buffer, pH 7), 1% α-naphthylacetate and Fast blue RR stain (2 mg/ml) were introduced.

The isoenzymatic fractions separated by electrofocusing were drawn and represented as zymograms. The stain intensity is indicated by hatching.

The phytochemical analyses were conducted on biological material dried at 40°C, powdered and then subjected to extraction with different solvents. Determination of polyholozidic content was realized in aqueous extract, the results being expressed as 'absent', +, ++ or +++, depending on reaction intensity. Methanolic extracts were processed to permit the quantification of flavones by colorimetric method (on account of complexation with $AlCl_3$) and of catechin-like polyphenolic derivatives by the colorimetric method, in the presence of 4-dimethyl-amino-antipyrine and ammonium persulfate, at pH = 8-9 (GRIGO-RESCU, STĂNESCU 1982).

For the determination of soluble protein the Lowry method (LOWRY et al. 1951) was used. The enzymatic extract was treated with Folin-Ciocâlteu solution. The extinctions were registered with a SPEKOL 20 spectrophotometer, at $\lambda = 500$ nm. The amounts of soluble proteins are expressed in mg/g fresh matter. These values are required to estimate the specific activities of the two hydroperoxidases.

The statistical analysis of the obtained data was performed using the method described by RAICU et al. (1973). The arithmetical mean ($\bar{x}$), the standard deviation (SD), the standard error of the mean (SE), the coefficient of variation (CV) and the standard error of the mean, expressed in % (SE %), were calculated.

Results and discussion

Quantitative analysis of catalase and peroxidase activities

As shown in Table 1, some differences are obvious between the two groups of plants of different sex. First, the male individuals have a greater catalase activity in relation to fresh biomass unit, as compared to females. The difference between mean values for males and females was 2.27 mg H_2O_2/g fresh matter. The peroxidase activity registered superior values in female plants, but the difference between mean values of female and male plants was smaller (0.9 mg H_2O_2/g fresh matter). The specific activities of the two enzymes were also different. Thus, the mean and the standard error of the mean of catalase were 4.88 ± 0.10 mg H_2O_2/mg protein, for the group of female plants, and 6.02 ± 0.10 mg H_2O_2/mg pro-

Table 1. Average values of the catalase and peroxidase activities and the amount of soluble proteins in male and female hemp plants

| Plant sex | n | Catalase | | | | | | | | Peroxidase | | | | | | | | Soluble proteins | | | |
| | | mg H_2O_2/g fresh matter | | | | mg H_2O_2/mg protein | | | | mg H_2O_2/g fresh matter | | | | mg H_2O_2/mg protein | | | | mg/g fresh matter | | | |
		$\bar{x} \pm SE$	SD	CV %	SE %	$\bar{x} \pm SE$	SD	CV %	SE %	$\bar{x} \pm SE$	SD	CV %	SE %	$\bar{x} \pm SE$	SD	CV %	SE %	$\bar{x} \pm SE$	SD	CV %	SE %
Female	20	27.62 ± 0.38	1.71	6.21	1.39	4.88 ± 0.10	0.45	9.22	2.04	10.53 ± 0.38	1.69	16.04	3.60	1.86 ± 0.02	0.10	5.37	1.07	5.659 ± 0.16	0.72	12.72	2.83
Male	15	29.89 ± 0.61	2.39	7.99	2.04	6.02 ± 0.10	0.39	6.47	1.66	9.63 ± 0.40	1.55	16.09	4.15	1.94 ± 0.05	0.21	10.82	2.57	4.961 ± 0.07	0.30	6.04	1.41

n = number of studied plants; $\bar{x}$ = mean; SE = standard error of the mean; SD = standard deviation; CV = coefficient of variation; SE % = standard error of the mean, expressed in %

tein, for the male individuals. The specific peroxidase activities registered values of 1.86 ± 0.02 mg H_2O_2/mg protein in pistillate plants and 1.94 ± 0.05 mg H_2O_2/mg protein in staminate plants. For both catalase and peroxidase, the specific activity was greater in males, but the increase is more important for catalase (1.14 mg H_2O_2/mg protein), while for peroxidase the increase is only 0.08 mg H_2O_2/mg protein.

The amount of soluble protein was greater in pistillate plants (5.659 ± 0.16 mg/g fresh matter). In staminate plants, these values were 4.961 ± 0.07 mg/g fresh matter, namely with 0.698 mg protein/g fresh matter smaller than those noted for female sexual phenotypes (Table 1).

The standard deviation gives indications on the spread of the observations around the mean. For the three analysed quantitative characters, the greatest concentration of the observations around the mean was registered for proteins. In this case, SD had the smallest values (0.30 for males, and 0.73 for females). The greatest deviations were noted for catalase: SD = 2.39 in males, and SD = 1.71 in females. The values of CV showed that the most variable character seems to be the peroxidase activity (CV = 16.09% for males, and CV = 16.04% for females). Besides that, the SE% had the highest values for this biochemical trait (4.15 for males and 3.60 for female plants) (Table 1).

The commentary on the obtained results must start from the fact that peroxidase activity is related to the developmental processes. Thus, in organogenesis, the role of peroxidase is frequently explained by the double function of this enzyme, involved both in oxidizing of some substrata and in auxine catabolism (LEGRAND, BOUAZZA 1991).The latter function enables the modulation of morphogenesis by peroxidase, as a result of intervention on endogenous hormonal balance. In hemp, as well as in other monoecious or dioecious plants, the gibberellins, auxins, ethylene and cytokinins have an important contribution to sex expression (MOHAN RAM, SETT 1982a, b, DURAND, DURAND 1984, CHAÏLAKHYAN 1985). These hormones generally intervene in the derepression of reglator genes, which enable the synthesis of specific proteins that control flower organogenesis. Because of its intervention in the regulation of IAA (indole-3-acetic acid) level, peroxidase has an indirect role in the sex-determining mechanism in hemp, more exactly in stamenogenesis and carpellogenesis. Hemp is one of the species in which a high level of IAA induces the female sex phenotypisation. The idea of a strong peroxidase activity associated with an increased auxine catabolism is generally accepted. Concerning catalase, the specific reaction catalysed by this enzyme is the direct degradation of the toxic H_2O_2 (the final product of biological oxidization), with release of water and oxygen, that is taken over to oxygenate the tissues. It seems, however, that the peroxidative activity of catalases (the reason for which the two enzymes are known as "hydroperoxidases") prevails in tissue. In the case of a smaller H_2O_2 concentration and of greater quantities of other substrata, catalase can use a hydrogen donor other than H_2O_2.

The isoesterase and isoperoxidase patterns

The isoenyzmatic patterns were done for single individuals. The differences between individuals of the same sex were not significant. Therefore, the schematic zymograms of izoesterases and isoperoxidases, for one male and one female, are compared in Figure 1. The esterases are hydrolases that catalyse the hydrolytic splitting of molecules of substrata at the level of esteric bonds, with formation of one alcohol molecule and one acid molecule. For both esterase (A) and peroxidase (B), the isoenzymatic spectrum is richer for staminate plants. Thus, in female plants, eight multiple isoesterase forms appear, six of them being placed in the domain of pH = 5.5-6.0 and the other two in the interval of pH = 6.5-7.0. The most active isoesterase band is placed in the weak acid domain, having pI (isoelectric point) at pH = 6.0. The eleven isoesterase forms of staminate plants are situated in the interval of pH = 4.5-7.2, the most active esterase forms having pI situated in the range of pH = 4.5-5.5, namely more acid that in the case of female plants. A specific aspect is the presence of three well outlined isoesterase bands at pH = 6.1-6.5 in male plants – bands that have no correspondence in the isoesterase pattern of pistillate plants.

Differences also exist for the isoperoxidase pattern. Thus, the female plant has, as in the case of esterase, fewer bands, two isoforms being in the range of pH = 4.5-5.5, one at pH = 7.4 and one at pH = 8.9 (extremely basic). The ten fractions of male genotypes were distributed in the following manner: seven at pH = 4.7-6.1 (acid), one isoform at pH = 7.4-7.6 (weakly basic) and two bands at the extremely basic value (pH = 8.9). The presence of four isoperoxidase fractions at pH = 5.8-6.1 confers a strong physiological advantage of male genotypes over the female genotypes. The isoesterase and isoperoxidase patterns reveal some differences at the metabolic level, related to a specific multigenic determinism. Peroxidase is distributed both in the cytosol and cell wall, as different genetic isoenzymatic forms (OKEY et al. 1997). Cell wall is regarded as the site of primary plant peroxidase activity (FRY 1986). Generally it is agreed that acid peroxidases (especially those found in the cell wall) intervene in lignin biosynthesis, while the basic ones (with cytoplasmic and vacuolar distribution) are involved in IAA catabolism, through a decarboxylation step (LIMAM et al. 1998). It is obvious (Figure 1) that, although there are fewer isoenzymatic bands in the female genotype, the bands associated with cell walls (active in the acid range) prevail in both analysed genotypes – a fact in accordance with data suggesting that cell walls are a principal site of plant peroxidases. Concerning acid peroxidases it is not clear if they are the products of different genes or if they are modified post-translational products of a small number of genes (ROS BARCELO et al. 1987). In hemp, like in other plant species, peroxidase has several isoforms, each with a well-defined role. The isoperoxidase pattern is complex, just this complexity being the element amplifying the difficulty to decipher all specific functions of this enzyme (CLEMENTE 1998, YUN et al. 1998). The genetic determinism of these isoforms is multigenic. If there are still unexplained details for hemp, for *Brassica napus*
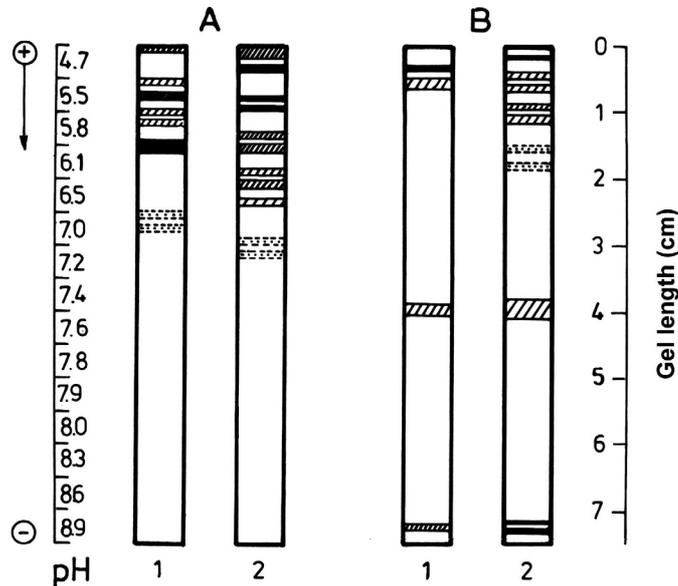
Figure 1. Zymograms of isoesterases (A) and isoperoxidases (B), in female (1) and male (2) of individuals *Cannabis sativa* L.

the existence of at least four distinct genes has been established (HAMED et al. 1998). The isoenzymes of *Petunia* are under the control of three genes, while in wheat isoperoxidases are controlled by different genomes and the environmental conditions do not modify the isoenzymatic pattern (HAMED et al. 1998).

Quantitative analysis of polyphenols, flavones and crude polyholozides

The data regarding the amount of some secondary metabolites, depending on sex and organ of the plant, were obtained from the individuals whose zymograms are presented in Figure 1. For every quantitative essay, two replications were made. Table 2 presents the average values of these phytochemical determinations, depending on sexual phenotype and on analysed organ, in dry and fresh matter. The comparative analysis of these results exhibits important differences. Thus, leaves from female plants have an increased level of polyphenols (1.560 mg%, expressed in catechin) and flavones (1.084 mg%, expressed in rutosid), while in male leaves polyphenols are not present, and the quantity of flavones represents 2/3 of the value for female leaves (0.680 mg%). Regarding the stem, no polyphenols were identified in the terminal (top) part of male plants. In female plants, however, their level was high (0.940 mg%, expressed in catechin). For flavones, the situation is inversed: in pistillate plants these compounds are not found,

Table 2. Average values registered for polyphenols, flavones and crude polyholozides, depending on sexual phenotype and on analysed organ in hemp

Plant sex	Organ	Polyphenols mg % catechin	Flavones mg % rutosid	Polyholozides
		Dry matter		
Male	terminal part of stem	0	0.460	++
Female	terminal part of stem	0.940	0	+
Male	leaves	0	0.680	+++
Female	leaves	1.560	1.084	++
Male	middle part of stem	0	0.525	+++
Female	middle part of stem	0	0	absent
Male	inflorescence	0	1.006	absent
		Fresh matter		
Male	leaves	0	0.340	+++
Female	leaves	0	0.525	+++
Male	inflorescence	0	0.444	+++

and in staminate plants their level was 0.460 mg%. In middle parts of stems, the results were negative for all three categories of tested compounds in female plants, whereas in male plants, polyphenols are lacking. The flavones of the rutosid type have a value of 0.525 mg%, and the crude polyholozides have a good representation (+++). Generally, higher levels of polyholozides were present in male plants (for example, ++ for terminal part of stem, +++ for leaves, +++ for middle part of stem, and for female plants, respectively: +, ++, absent). The quantitative analyses conducted in fresh matter were negative for polyphenols for all tested organs, both for male and female plants, but the flavones and crude polyholozides were present, the latter in high levels (+++) in all samples. Thus, between sexual phenotypes as well as between the organs of hemp plants, visible differences exist. It is also important that in the fresh and dry matter of the male plants, polyphenols were absent – an aspect possibly related to the fact that in hemp species, in which a high IAA level favours the phenotypisation of female sex, the capacity to degrade IAA is counterbalanced by auxine protectors (phenols).

As in the case of other secondary metabolites, the hemp callus was unable to synthetize polyphenols and flavones (TRUȚĂ, unpublished), which is in accordance with results of other studies (BRAEMER et al. 1985, BRAUT-BOUCHER et al. 1981, GILLE 1996).

Conclusions

In this study, the isoenzymatic pattern of esterase and peroxidase is richer in hemp male plants, as compared to female plants. For both analysed sexes, the isoperoxidase bands localized in acid domain are prevalent. The relative and specific activity of catalase have more reduced values in female plants. The specific peroxidasic activity is greater in male plants. The average level of soluble protein was higher in female plants. Significant differences are registered, depending not only on sex, but also on tested organ in the same plant, in respect of level of polyphenols, flavones and polyholozides. In male plant, polyphenols were absent.

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