INDIRECT CRITERIA TO DETERMINE THE MICROSPORE DEVELOPMENTAL STAGE IN HEMP ANTHERS

CRITERII INDIRECTE PENTRU DETERMINAREA STADIULUI DE DEZVOLTARE A MICROSPORILOR ÎN ANTERELE CÂNEPEI

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Abstract. *The study was performed to establish if the length of the male floral buds may be considered as an indirect criterion to determine the microspore developmental stage in Cannabis sativa L. anthers. The analyzed cultivars show a normal microsporogenesis, in the climatic chamber growing conditions, with defects in the heterotypic division. There is a significant interaction between the cultivar, floral bud size group and the microspore developmental stage. Yet the male floral buds of the same size, but within different cultivars, don't have the microspores in the same developmental stage.*
Key words: *hemp, microsporogenesis, microspore, floral bud*

Rezumat. *Studiul a fost efectuat pentru a stabili dacă lungimea bobocilor florali masculi poate fi considerată un criteriu indirect pentru determinarea stadiului de dezvoltare a microsporilor în anterele de Cannabis sativa L. Cultivarele analizate prezintă o microsporogeneză normală în condițiile de creștere din laborator, cu defecte ale meiozei în cadrul diviziunii heterotipice. Se constată o interacțiune distinct semnificativă între cultivar, grupa de dimensiune a bobocilor florali și stadiul de dezvoltare al microsporilor. Totuși, bobocii florali masculi de aceeași dimensiune dar din cadrul a diferite cultivare, nu conțin microspori în același stadiu de dezvoltare.*
Cuvinte cheie: *cânepă, microsporogeneză, microspor, boboc floral*

INTRODUCTION

Hemp microsporogenesis takes place normally, with differences between different plants, different floral buds within an inflorescence, and different cells of the same floral bud (Xin et.al., 2008). Some factors such as the hybrid origin of the plant, parthenogenetic propagation, growing conditions, (Medwedewa, 1935), parasite attacks (Asanova, 2002) may determine specific irregularities in the course of microsporogenesis.

During male sexual cells formation, there is a favorable moment for androgenesis induction in plants. As a general rule, this sensitive period of microspores ranges between the uninucleate stage and bicellular pollen around the first mitosis (Segui-Simarro, 2010).

To avoid cytological identification of the microspore developmental stage, indirect criteria can be used, such as the size of the floral bud, the size and color of the anther (Gheorghiță and Nicuță, 2005).

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MATERIAL AND METHOD

Plant material: seven hemp cultivars (*Zenit, Denise, Diana*, *Lovrin 110*, *Lovrin 200*, *SF 200* şi *ZF 314*) were grown at 27-29ºC temperature and 16 hours light / 8 hours dark photoperiod until they reached 2 months. After 2 months the photoperiod was adjusted to 12 hours light / 12 hours dark, for flowering stimulation. Plants were cut above the third pare of true leaves and above the third pare of sprigs leaves for keeping the plants height into the limits imposed by the climatic chamber.

Cytogenetic investigations: male floral buds were harvested at emergence, beginning of flowering and full flowering, measured and classified in 4 size groups, (0.1-2 mm, 2.1- 4 mm, 4.1- 6 mm, 6.1- 8 mm) then fixed in Carnoy I solution and kept at 4ºC. From a male floral bud, an anther was excised and maintained in Carr dye for 30 minutes at room temperature. The anther was displayed using the squash technique and images were digitally retrieved with a built in camera Motic B Series optic microscope, and Motic Images 2.0 program. Inside each cultivar, 5 floral buds were analyzed for each bud size group.

Meiotic stages were analyzed and chromosomal aberrations counted separately. Data were expressed as percentages and arc-sin transformed before ANOVA, to normalize the distribution. Duncan's test was effectuated. For each cultivar, the experiment was designed after the randomized blocks method, 4 x 7, N=140, in 5 replications.

RESULTS AND DISCUSSIONS

Microsporogenesis

Archesporial cells, closely adjoined one another, have irregular, polygonal shape, with a large nucleus and a loosely arranged chromatin. Repeated mitotic divisions that lead to microsporocytes formation take place asynchronously. The sporogenous tissue consists of larger cells than the archesporial ones, with irregular shape (fig. 1, a), and visible nucleolus in interphase fig. 1, b).

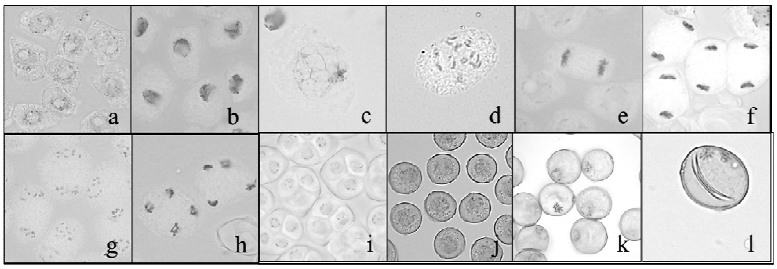

Fig. 1 - Microsporogenesis in *Cannabis sativa* L. a) sporogenous cells; b) interphase; c) prophase I, leptonema; d) prophase I, diakinesis; e) anaphase I; f) telophase I with condensed nuclei; g) prophase II; h) anaphase II; i) tetrad; j) microspores with a double membrane; k) first pollen mitosis, metaphase; l) first pollen mitosis, telophase.

Prophase I is normal (fig. 1, c, d), at diakinesis the diploid chromosome number being easy to determine (fig. 1, d). Metaphase I is characterized by chromosomes spread along the equatorial plate, which then separate and head to

the nuclear spindle poles in anaphase I (fig. 1, e). They condense in easily stainable nuclei, of the same size, towards the end of telophase I (fig. 1, f).

After the fragmentation of nucleoprotein filaments in 10 chromosomes during prophase II (fig. 1, g), the second meiotic division initiates. In anaphase II, the orientation of division spindles is bilateral (fig. 1, h). At the end of meiosis II tetrads are formed, both tetrahedrally and tetragonally arranged (fig. 1, i), the four microspores always being mononuclear. The tetrad membrane disintegrates releasing the young microspores which will shortly form a double membrane and germinative pores (fig. 1, j). They become polarized and the first pollen mitosis begins (fig. 1, k, l).

There have been identified chromosomal aberrations during meiosis only in the heterotypic division, represented by lagging chromosomes and micronuclei formation in metaphase I, lagging chromosomes, simple or multiple bridges in anaphase I, lagging chromosomes, micronuclei formation and discontinued bridges in telophase I.

Intra cultivar analysis. Zenit cultivar has microspores in male floral buds ranging from 2.1 to 6 mm, with significant superior values for 4.1-6 mm bud size group compared with 2.1-4 mm size group ($DS_{5\%}$=22,06) (tab. 1). Uninucleate pollen has significant values for 6.1-8 mm bud size group compared with 4.1-6 mm bud size group ($DS_{5\%}$=21,72).

Table 1

Microspore development stage depending on male bud size, for Zenit cultivar

Bud size (mm)	Development stage						
	Premeiosis	Meiosis1	Meiosis2	Tetrad	Microspore	Uninucleate pollen	Binucleate pollen
0 - 2	84.3 a	7.39 d	0 d	0 d	0 d	0 d	0 d
2,1 - 4	22.93 cd	39.58 c	16.13 d	7.47 d	9.54 d	0 d	0 d
4,1 - 6	0 d	0.36 d	3.24 d	0 d	67.79 ab	16.79 d	3.3 d
6,1 - 8	0 d	0 d	0 d	0 d	0 d	82.12 ab	7.88 d

Difference between each two variants is not significant if followed by the same letter or group of letters; $s_{\square}$ = 6,96; $DS_{5\%}$ = 19,48 – 24,45.

Denise cultivar has microspores in anthers excised from 2.1-4 mm male buds (tab. 2), and uninucleate pollen starting with 2.1-4 mm bud size group to 8 mm buds.

Table 2

Microspore development stage depending on male bud size, for Denis cultivar

Bud size (mm)	Development stage						
	Premeiosis	Meiosis1	Meiosis2	Tetrad	Microspore	Uninucleate pollen	Binucleate pollen
0 – 2	54,12abcd	35,87de	0e	0e	0e	0e	0e
2,1 - 4	0e	0e	9,04e	8,61 e	74,09abc	2,49 e	0e
4,1 - 6	0e	0e	2,29e	0e	0e	84,45a	2,91e
6,1 - 8	0e	0e	0e	0e	0e	81,55ab	8,45e

Difference between each two variants is not significant if followed by the same letter or group of letters; $s_{\square}$ = 11,81; $DS_{5\%}$ = 33,06 – 40,98.

Values corresponding to uninucleate pollen in 2.1-4 mm bud size group are significantly inferior to those in 4.1-6 mm bud size group ($DS_{5\%}$=38,85) and 6.1-8 mm respectively ($DS_{5\%}$=38,38). There are no statistical differences between 4,1-6 mm male buds and 6,1-8 mm male buds regarding the presence of uninucleate pollen ($DS_{5\%}$=33,06).

Table 3

Microspore development stage depending on male bud size, for Diana cultivar

Bud size (mm)	Development stage						
	Premeiosis	Meiosis1	Meiosis2	Tetrad	Microspore	Uninucleate pollen	Binucleate pollen
0 – 2	47,8 b	42,19bc	0 g	0 g	0 g	0 g	0 g
2,1 - 4	0 g	24,11de	22,71def	8,45efg	38,15bcd	0 g	0 g
4,1 – 6	0 g	0 g	3,43 g	0 g	0 g	83,73 a	3,39 g
6,1 - 8	0 g	0 g	0 g	0 g	0 g	78,2 a	11,79efg

Difference between each two variants is not significant if followed by the same letter or group of letters; $s_{\square}$ = 5,68; $DS_{5\%}$ = 15,92 – 19,73.

Diana cultivar shows microspores only in 2.1-4 mm bud size group and uninucleate pollen in 4.1-6 mm and 6.1-8 mm buds, with no significant difference between the two bud size groups (DS5%=15,92) (tab. 3).

In the case of ZF 314 microspores from 4.1-6 mm bud size group have significant superior values than 2.1-4 mm bud size group ($DS_{5\%}$=23,83) (tab.4). Uninucleate pollen is encountered starting with 4.1 mm buds, with significant superior values for the 6.1-8 mm bud size group ($DS_{5\%}$=23,83).

Table 4

Microspore development stage depending on male bud size, for ZF 314 cultivar

Bud size (mm)	Development stage						
	Premeiosis	Meiosis1	Meiosis 2	Tetrad	Microspore	Uninucleate pollen	Binucleate pollen
0 – 2	63,74 ab	25,91cde	0,81 f	0 f	0 f	0 f	0 f
2,1 - 4	0 f	28,86 cd	34,27c	16,67cdef	14,76cdef	0 f	0 f
4,1 - 6	0 f	0 f	7,7 def	0 f	63,52 ab	19,25cdef	0,63f
6,1 - 8	0 f	0 f	0 f	0 f	0 f	77,06 a	12,74cdef

Difference between each two variants is not significant if followed by the same letter or group of letters; $s_{\square}$ = 7,4; $DS_{5\%}$ = 20,72 – 25,68.

Table 5

Microspore development stage depending on male bud size, for SF 200 cultivar

Bud size (mm)	Development stage						
	Premeiosis	Meiosis1	Meiosis2	Tetrad	Microspore	Uninucleate pollen	Binucleate pollen
0 – 2	50,85 a	35,23abcd	5,32 f	0 f	0 f	0 f	0 f
2,1 - 4	0 f	4,15 f	24,58 abcdef	22,47 bcdef	40,87abc	0,97 f	0 f
4,1 - 6	0 f	0,51 f	3,38 f	0 f	34,64 abcde	46,66 ab	6,22 f
6,1 - 8	0 f	0 f	0 f	0 f	0 f	81,8	8,06 f

Difference between each two variants is not significant if followed by the same letter or group of letters; $s_{\square}$ = 8,63; $DS_{5\%}$ = 24,2 – 29,9.

For SF 200 cultivar, analyzed slides indicate the presence of microspores in male floral buds ranging from, 2.1 to 6 mm, with no significant difference between the two bud size groups ($DS_{5\%}$=25,5).

Uninucleate pollen has significant superior values for 6.1-8 mm bud size group from 4.1-6 mm group ($DS_{5\%}$=25,5) and 2,1-4 mm group respectively ($DS_{5\%}$=29,3) (tab. 5).

Lovrin 110 has microspores in 2,1-4 mm and 4,1-6 mm bud size groups, with no significant difference between the observed values ($DS_{5\%}$ = 24,93) (tab. 6). The microspores continue to develop into uninucleate pollen starting with the anthers from 2,1-4mm bud size group.

Table 6

Microspore development stage depending on male bud size, for Lovrin 110 cultivar

Bud size (mm)	Development stage						
	Premeiosis	Meiosis1	Meiosis2	Tetrad	Microspore	Uninucleate pollen	Binucleate pollen
0 – 2	38,81 ab	29,02 abcd	25,32 bcde	0 e	0 e	0 e	0 e
2,1 - 4	0 e	5,85 de	23,38 bcde	25,33 bcde	38,77 abc	4,05 de	0 e
4,1 - 6	0 e	0 e	0 e	9,55 de	23,91bcde	52,54 a	8,42 de
6,1 - 8	0 e	0 e	0 e	0 e	0 e	80,2	9,67 de

Difference between each two variants is not significant if followed by the same letter or group of letters; $s_{\square}$ = 7,99; $DS_{5\%}$ = 22,37 – 27,72.

High values for uninucleate pollen in 6,1-8 mm bud size group are significantly superior to those recorded for 4,1-6 mm ($DS_{5\%}$=22,37) and 2,1-4 mm bud size groups ($DS_{5\%}$=27,08).

Lovrin 200 shows microspores in 2,1-4 mm and 4,1-6 mm bud size groups, with significant superior values for the last group ($DS_{5\%}$ = 23,8). Uninucleate pollen is encountered in 4,1-6 mm and 6,1-8 mm bud size groups, with significant superior values in 6,1-8 mm ($DS_{5\%}$ = 22,9) (tab. 7).

Table 7

Microspore development stage depending on male bud size, for Lovrin 200 cultivar

Bud size (mm)	Stadiul de dezvoltare						
	Premeiosis	Meiosis1	Meiosis2	Tetrad	Microspore	Uninucleate pollen	Binucleate pollen
0 – 2	82,26 a	7,74 e	0 e	0 e	0 e	0 e	0 e
2,1 - 4	23,18 cde	37,76 bc	17,66 cde	7,69 e	11,13 e	0 e	0 e
4,1 - 6	0 e	0 e	2,55 e	0 e	50,79 b	34,84 bcd	2,92 e
6,1 - 8	0 e	0 e	0 e	0 e	0 e	79,74 a	10,26 e

Difference between each two variants is not significant if followed by the same letter or group of letters; $s_{\square}$ = 7,51; $DS_{5\%}$ = 21,02 – 26,05.

Depending on the presence of microspores and uninucleate pollen, for each male bud size group, we classified the analyzed cultivars into three classes (tab. 8).

Table 8

Male floral bud size and microspore developmental stage for the 7 analyzed *Cannabis sativa* L. cultivars

Class no.	Microspores in group size	Uninucleate pollen in group size	Cultivars
1	2,1 – 4 mm	4,1 – 6 mm + 6,1 – 8 mm	Denise, Diana
2	2,1 - 4 mm + 4,1 – 6 mm	6,1 – 8 mm	SF 200, Lovrin 110
3	4,1 – 6 mm	6,1 – 8 mm	Zenit, ZF 314, Lovin 200

CONCLUSIONS

1. Hemp pollen formation is as normal in the experimented growing conditions;

2. Male floral buds of the same size but from different cultivars do not necessary have microspores in the same developmental stage;

3. The male floral bud size can be considered a indirect criteria for determining the microspore developmental stage.

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