ABSTRACT

Key words: hemp, microsporogenesis, androgenesis

Hemp (*Cannabis sativa* L.) has a growing economical importance, because of the various utilizations in alimentation, textiles, plastics, constructions and medicine.

The improvement of hemp cultivars traits may be eased by modern vegetal biotechnologies, of which, the utilization of experimental androgenesis for double haploid production is important for breeding and for future genetic studies of the species.

The doctoral thesis with the title: "Microsporogenesis particularities and the induction of experimental androgenesis in hemp (*Cannabis sativa* L.)" is structured in two parts and contains eight chapters.

The first part presents a bibliographical study and contains the description of the *Cannabis sativa* L. species, general data regarding microsporogenesis and androgenesis induction.

The second part contains a presentation of the institutional framework were the researches were performed, the objectives, biological material, research methods and personal results.

Chapter I - *Cannabis sativa* l., species description, presents the hemp history and utilizations, the morphology, systematic and phylogeny, as well as a synthesis of the genetic and breeding studies undertaken at this species.

Chapter II - Researches regarding the microsporogenesis in *Cannabis sativa* L. presents general and particular aspects with respect to male gametes formation and natural defects of the microsporogenesis in hemp.

Chapter III - Researches regarding the androgenesis induction in *Cannabis sativa* L. comprises general aspects regarding the methods for experimental androgenesis induction, the factors that influence androgenesis in plants, as well as previous studies on androgenesis induction in hemp.

Chapter IV - Objectives, material and research method

In order to identify the optimal *in vitro* conditions needed for the manifestation of the hemp cultivar androgenetic potential, there were established the following secondary objectives:

- To establish some indirect criteria for microspore developmental stage determination;
- To establish the hemp cultivars that have high androgenic potential;
- To establish the hemp cultivars that have high regenerative / embryogenic potential;
- To establish the optimal phytohormones combination for androgenesis induction;
- To establish the optimal phytohormones combination for embryogenesis / caulogenesis induction in hemp.

The biological material was comprised of five hemp cultivars. Three of them were monoecious, were created at the RDAS Secuieni and two cultivars, dioecious were created at the RDAS Lovrin.

Male floral buds were harvested in different developmental stages (occurrence of male floral buds, beginning of flowering and full flowering), measured and grouped in four dimension categories. After fixation, one anther from every bud was analyzed. All meiotic phases were studied and associated with the length of the male floral buds, to establish some indirect criteria for the determination of microspore development stage.

Floral buds with the microspores in the optimal developmental stage for androgenesis induction were harvested in the morning and sterilized, anthers collected aseptically and inoculated in Erlenmeyers (100 ml) containing each 20 ml culture medium, supplemented with different phytohormonal combinations.

Inoculated anthers were incubated in dark, at a temperature of 28^{o}C for 3 weeks, then were transferred in the climatic chamber, at a temperature of 25^{o}C and photoperiod of 16 hours light / 8 hours dark. The following observation were made: callus induction, callus proliferation, anther color during incubation, callus color during incubation, callus color when transferred to light and type of callus, in order to establish the hemp cultivars with high androgenic potential and the optimal phytohormonal combination for androgenesis induction.

Callus induced in anther culture was sectioned in equal pieces and transferred to organogenesis / embryogenesis induction medium in 100 ml Erlenmeyers with 20 ml culture

medium supplemented with different phytohormonal combinations. Callus was maintained at 25^{o}C and photoperiod of 16 hours light / 8 hours dark.

Organogenesis / embryogenesis was appreciated depending on the percentage of callus with necrosis, rizogenesis or embryogenesis.

Chapter V - Associations between microspores development stages and the length of male floral buds in *Cannabis sativa* L.

Male floral buds length may be considered an indirect criterion for microspore development stage development, but this criterion must be verified for each *Cannabis sativa* L. cultivar, at the initiation of anther culture, because anthers do not necessarily contain microspores in the same developmental stage, for male floral buds of same length but from different cultivars.

In general, uninucleate microspores were identified in the anthers of the male floral buds with the length between 4,1 cm and 6 cm.

Chapter VI – Androgenesis induction in anther culture of *Cannabis sativa* L.

Silvana and Denise cultivars were characterized by the highest callus induction frequencies, while Diana cultivar had the lowest callus induction capacity, on different phytohormonal combinations.

Callus proliferation is different, depending on cultivar. The best proliferation was observed for Denise cultivar, followed by Silvana. Diana had the poorest proliferation of the androgenetic structures induced in anther culture.

A real connection was observed between the percentage of anthers that generated callus and callus proliferation. However, there are cases when a cultivar with smaller callus induction frequencies had maximum proliferation.

In general, good callus induction and proliferation was observed on MS culture medium suplimented with 1 mg/l 2,4D + 2 mg/l KIN. Good results were also observed on MS culture medium suplimented with 2 mg/l AIA + 1 mg/l KIN. Higher concentrations of 2,4D or lower concentrations of AIA inhibited callus induction and proliferation.

Chapter VII - Organogenesis / embryogenesis induction from induced callus in anther culture of *Cannabis sativa* L.

Zenit cultivar did not presented necrosis on any of the five tested phytohormonal combinations, the most affected cultivar being Lovrin 110, especially on MS medium supplemented with 0,5 mg/l AIA + 1 mg/l BAP. Besides, the utilization of BAP in small

concentrations as cytokinin source in combination with 0.5 mg/l AIA determines callus necrosis with higher frequencies on the majority of the studied cultivars, except for Zenit.

Rhizogenesis was observed for all studied cultivars, Lovrin 110 being the most responsive from this point of view. Utilizing KIN as cytokinin source, on a phytohormonal balance in favor of the cytokinins, promotes root formation, the frequency of this phenomenon being higher at elevated KIN concentrations. The same tendency of rhizogenesis frequency increment was also observed when utilizing BAP.

The cultivar has a significant effect on embryogenic callus presence. In our experimental conditions, the callus induced by Zenit cultivar was characterized by embryogenesis at higher frequencies, whilst for Denise cultivar the presence of such structures was not observed. The utilized cytokinins influence the presence of pro-embryonic structures. A stimulatory effect from din point of view was observed in the presence of BAP (2mg/l) or in the presence of 1mg/l KIN + 1 mg/l BAP. Caulogenesis was not observed.

Chapter VIII - Morphogenetic reaction of *in vitro* cultivated anthers.

Anthers presented a large variety of colors during incubation. Dark brown anthers did not generate callus, or the induced callus died, with no proliferation.

Androgenetic structures were observed on green, yellowish-green anthers, with or without brown or yellowish-brown spots. Subsequently anther color changed and become light-brown or brown.

After approximately 14 days of culture, the first androgenetic structures appeared, different in frequency, color and type, depending on cultivar and phytohormonal combination. Differences in anther reaction were observed even in the same culture vessel.

During incubation, callus was cream-colored, occasionally white. After the transfer to light, the callus had different development, depending on cultivar and phytohormonal combination. In general callus maintains the compact structure, even if a difference of color or proliferation was observed.

After the transfer on differentiation / regeneration mediums, some of the compact callus becomes friable, granular. Proliferation was observed for the majority of transferred structures. Under 0.5 mg/l AIA and different cytokinins influence, the callus remains, cream-colored, green or brown, or changes its color. Callus with different colored areas was observed.

Green callus was characterized by meristematic centers. Some of the transferred structures become friable, with granular aspect or not. There were cases when the same callus

presented different types of areas, the embryonic callus being observed only on friable, green callus. When rhizogenesis was observed, the phenomenon was intense on cream-colored or brown callus. When transferring the callus so that the roots were in contact with the culture medium, the roots developed rapidly, especially when the culture medium was supplemented with KIN as cytokinin source.

Caulogenesis was not observed for any cultivar or phytohormonal combination tested.