eJBio

Electronic Journal of Biology, 2013, Vol. 9(2):29-36

Capsaicin - Inhibitory Factor for Somatic Embryogenesis in Pepper Anther Culture

Liljana Koleva Gudeva[1,*], Rubin Gulaboski[1], Emilija Janevik-Ivanovska[2], Fidanka Trajkova[1], Viktorija Maksimova[2]

1 Faculty of Agriculture, Goce Delcev University, Krste Misirkov bb, PO box 201, 2000 Stip, R. Macedonia;

2 Faculty of Medical Sciences, Goce Delcev University, Krste Misirkov bb, PO box 201, 2000 Stip, R. Macedonia.

* Corresponding author. Tel: +389(0)32 550 627; Fax: +389(0) 390 700; E-mail: liljana.gudeva@ugd.edu.mk

Abstract

The androgenesis of pepper (*Capsicum annuum* L.) is very limited process and it is followed by many inhibitory factors such as: pepper genotype; the structure and the stadium of microspores; the genetic predisposition for somatic embryogenesis; the hormonal regulation under *in vitro* condition; and growth conditions. Pungency in *Capsicum* fruits is due to the accumulation of the alkaloid capsaicin and its analogs. The biosynthesis of capsaicin is restricted to the genus *Capsicum* and results from the acylation of an aromatic moiety, vanillylamine, by a brached-chain fatty acid. The inhibitory influence of secondary metabolites, including capsaicin, is not explored enough, although in the literature there are data that some pepper genotypes as bell-shape and sweet ones have higher androgenic potential than the hot genotypes.
The results from our research work, performed on nine pepper varieties which differ in pungency, showed that the androgenic potential of pepper anthers culture is dependent on the capsaicin content in the pepper fruits. Most probably the genetic predisposition for synthesis of capsaicin as secondary metabolite beside all the other factors is also inhibitory trait of the somatic embryogenesis.
Our research showed that there is negative correlation between the capsaicin content of fruits of all the varieties under investigation and the percentage of embryogenic anthers and the number of formed embryos per 100 anthers. The hottest cultivar Feferona showed neither androgenesis nor callus formation, as compared to the other two hot cultivars where the callus formation is the main process. The callus formation of the sweet cultivars is moderate, while the androgenic response is poor to fair. The sweetest cultivar Féherözön showed excellent androgenic response with 31.09% androgenic and only 3.92% callused anthers. The aforementioned facts gives an idea that the capsaicin has an inhibitory effect in *in vitro* conditions and the more pungent cultivars possess less androgenic potential as compared to the sweet and bell-shaped ones.

Keywords: *Capsicum annuum* L., pepper anther culture, somatic embryogenesis, secondary metabolites, androgenic response.

1. Introduction

Pepper (*Capsicum*) was among the earliest domesticated plant genera, based upon archelogical evidence from Central America dating back at least 7000 years [1]. In 1876, the alkaloid capsaicin was identified as the compound responsible for the characteristic pungency in pepper [2]. Within the pepper fruits, capsaicin and its alongs, known collectively as capsaicinoides [3], are synthesized in the epidermal cells of the placental dissepiment beginning approximately 20 days post-anthesis, and accumulate in pockets or blisters along the epidermis [2]. Biosynthesis of this group of compounds is unique to the *Capsicum* genus, and has driven the domestication of several species including *C. annuum*, *C. frutiscens* and *C. chinense*, which are now valued for use as vegetables, spices, and medical and industrial purposes [4]. The capsaicin (8-Methyl-N-vanillil-trans-6-noneanamide) is the most pungent substance from the group of compounds called capsaicinoids that can be isolated only from genus *Capsicum* [5].

Capsaicinoides are complexes of related components, benzilamin derivates, and the five general representatives are: capsaicin (69% represented in the group of capsaicinoides); dihydrocapsaicin (22% represented in the group of capsaicinoides); nordihydrocapsaicin (7% represented in the group of capsaicinoides); homocapsaicin and homohydrocapsaicin (both represented with 1% in the group of capsaicinoides). From all the capsaicinoides only capsaicin and dihydrocapsaicin with 80%-90% are responsible for the chilli taste in pepper [6]. Capsaicinoides are produced by the condensation of vinillyamine, derived from phenylalanine, with a branched-chain

fatty acid, derived from valine or leucine [3,7]. The synthesis of capsaicinoides is genetically inherited characteristic which is expressed also in *in vitro* conditions through alternative pathways [8-10].

The capsaicin is strong and stable crystal alkaloid, which does not change the properties on cold and heat, which is the reason for keeping the original strength after a long period of time with heating or freezing. Although, it is without colour, taste and odour, the capsaicin is one of the hottest known substances, and according to De Witt [11], the human palate senses it in dilution 1:17 000 000. The different content of capsaicinoids in pepper gives different spiciness of the fruit, so there are hot and sweet ones [6]. The clinical research showed that the biological potential of capsaicin comes from its incredibly strong and stable structure as an alkaloid with various inhibitory effects: analgetic and pain restrictor [12-19]; antimicrobial [20]; antibacterial [15,21-23]; anticarcinogenic [15,24]; carcinogenic [25,26],; anesthetic [11,12,14,]; cytostathic and homotherapic [27,28].

Since pepper is a recalcitrant species, moderate results can be achieved in tissue culture. *In vitro* anther culture seems to be only exception under these conditions. There are several factors affecting androgenesis in many species, such as genotypes [29,30]; growth of donor plants, pre-treatments of anthers [8,31] and composition of medium [32-34].

It is well known that the pungent pepper cultivars are regenerating more difficult *in vitro* conditions as compared to the sweet ones. Bell-shape peppers were generally characterized by fair or good androgenic response in the anther culture of Mitykó and Fary [35]. In 1993 Quin and Rotino [36] and later in 1994 Ltifi and Wenzel [37] in experiment of pepper anthers culture from sweet and hot varieties concluded that the hot varieties possess either lower androgenetic potential or there is no embryo formation at all. The authors commented this phenomenon as result of the effects of different growth regulator combination on the androgenic response and on different genotype effect. The possibility of capsaicin and its analogs capsaicinoides inhibitory effect *in vitro* conditions is not studied at all, but it is a question that can give many opportunities for further research work.

Haploid and spontaneous diploid plant production from anther culture is a well-developed and useful tool in practical plant breeding as well as in basic research. The first *in vitro* haploid pepper production via anther culture was obtained in 1973 by Wang et al. [38]. Haploid morphogenesis in *Capsicum* has been reported by George and Narayanaswamy [39] and Kuo at al. [40], even though the production of haploid individuals had been very low. Later in 1981, a reproducible anther culture method was developed by Dumas de Valux, et al [41]. Since only a low number of haploid plantlets were regenerated from excised anthers, further investigations were carried out, regarding not only the composition of the culture medium, but also other factors affecting the frequency of haploid induction [28,29].

The inhibitory effect of the capsaicin is proved in many physiological processes as antibacterial and antimicrobial, but regarding the inhibitory influence in *in vitro* cultures the knowledges are quite insufficient. The aim of this study was to determine the effects of the capsaicin content on androgenetic potential of nine different pepper cultivars, starting from the literature data that sweet pepper cultivars have greater androgenic capacity that the pungent ones.

2. Materials and Methods

Plant material and donor - plant growth conditions

Nine varieties of pepper (*Capsicum annuum* L.), different in pungency, were used for anther-donor plant materials: Feferona (long, hot type), Slatko Luta (long, moderate hot type), Vezena Luta (long, hot type), Sivrija (long, sweet type), Zlaten Medal (sweet spice type), Kurtovska Kapija (sweet spice type), Californian Wonder (bell-shaped, sweet type), Rotund (tomato-shaped, sweet type), and Féherözön (Hungarian wax-bell-shaped, sweet type). Plants were grown in pots, under greenhouse condition. Regular cropping practices regarding fertilization and irrigation were conducted during the whole vegetative period. Anther-donor mother plants were used during the 4 weeks after the first flower buds had appeared. Flower buds were harvested when the corolla was of the same length as the calyx or little longer. The fruits for analysis of capsaicin were collected in full botanical maturity, on the end of the plant vegetation, when the synthesis of the capsaicin is the highest.

Measurement of capsaicin content

The plant material was collected *in vivo* conditions from fruits of the nine varieties of pepper grown in greenhouse. The plant samples of fully ripen pepper fruits for capsaicin analyses were dried to air dry weight (at room temperature, 6-7 days). The additional humidity was corrected with drying of the samples until stable weight, in an oven on 55^{o}C for 5 hours. Dried pepper fruits were crushed in a coffee grinder with the distribution of particle size from 0.25 to 0.50 mm. The ethanol extract (oleorasin) from dried and grinded fully ripen pepper fruits (0.1 – 0.5g) was done with 96% vol C_2H_5OH in water bath on 40^{o}C for 5 hours. Subsequently, the ethanol extract of the capsaicin was filtrated through a 0.45 μm filter by water vacuum filtration. The extract was adequately diluted for measuring on an instrument. The absorbance of the total capsaicin in the ethanol extract was measured on spectrophotometar (Varian UV-VIS, Caryso Series) at wave-length of 281 nm. The standard curve was prepared with serial of standard solutions (from 0.02 to 0.1 mg·ml^{-1}) of capsaicin from *Capsicum* fruits (8-

Methyl-N-vanillil-trans-6-noneanamide, $C_{18}H_{27}NO_3$, FW 305.4, Sigma, M2028) and the coefficient of linear correlation of the standard curve was R^2=0.998 (y=9.7734x+0.1409) (Figure 1A and 1B).

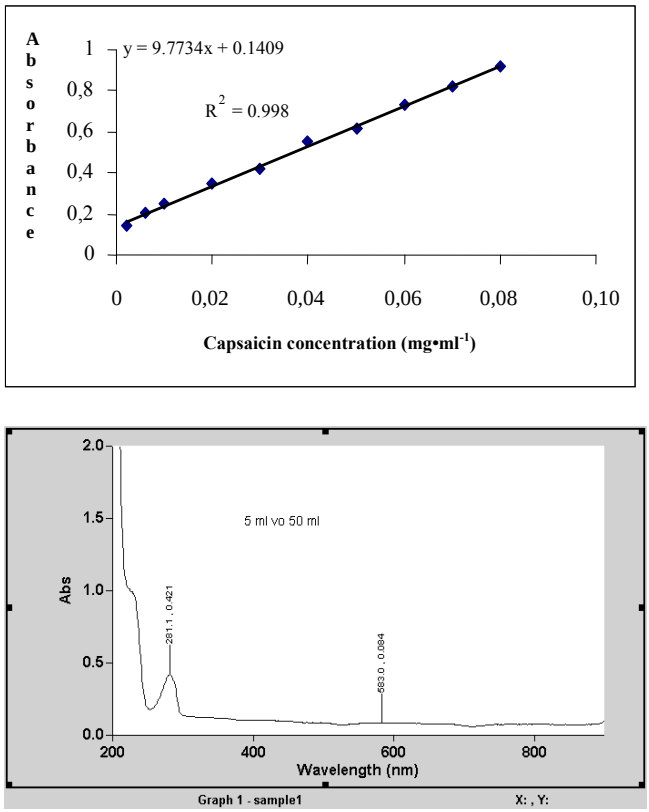

Figure 1. (Above) Capsaicin standard curve at 281 nm; (Bellow) UV-VIS spectar of capsaicin standard (Sigma) with typical pick at 281 nm.

Anthers culture of pepper

Immature buds from the pepper cultivars under study were used as starting material for androgenesis. Usually, this is the phase of flower bud when the length of sepals and petals are the same and the free ends of the anthers are slightly anthocyanated. The buds were rinsed with distilled water, surface sterilized with 70% ethanol for 15 seconds, and 5% calcium hypochlorite with 2-3 drops Tween 20 for 20 minutes and rinsed several times with sterile water. In order the division stadium of microspore to be determined, the anthers were dyed with aceto carmine for few minutes and then were squashed and observed under microscope. Collected buds must contain anthers with microspores at the stage of first pollinic mitosis, or just before the division.

The preparation of the aceto carmine for determination of microspores stadium was done as follows: 1g of carmine was resolved in 45 ml glacial acetic acid, 55ml of distilled water was added and put to boil for 5 minutes. The solution was left to cool, to filtrate and 1-2 drops of iron hydroxide were added for intensification of the colour. A drop of aceto carmine was placed on the isolated anthers. After few minutes the anthers were macerate on the microscopic glass slide, the slide was placed under the microscope and we observed in which stadium were the microspores.

Culture media and conditions

Fifteen anthers were plated on to 5 cm diameter Petri dishes, the concave side touching the Cp inductive medium [13], supplemented with 0.01 $mg \cdot l^{-1}$ Kn and 0.01 $mg \cdot l^{-1}$ 2,4 D. The first 8 days the cultures were incubated at 35±2°C in darkness, and the following 4 days at 25±2°C, with a photoperiod of 12 hours light at 30-40 µmol m^{-2} s^{-1}. After 12 days of induction in Cp medium anthers were transferred to R_1 medium [13], supplemented with 0.1 $mg \cdot l^{-1}$ Kn and placed in a growing chamber at 25±2°C, with a photoperiod of 12 hours light at 30-40 µmol m^{-2} s^{-1}. Embryos emerging from the anthers were transferred into test tubes onto V_3 hormone-free medium for further development into plantlets [13]. Well-developed plantlets were planted into pots on sterile mixture of peat : sand : perlite (1:1:1) and were placed in acclimatization chamber.

Process of somatic embryogenesis by anther culture

In order to identify and select the starting material at the onset of the culture, initial analyses of the flower bud morphology, the size of anthers and the developmental stage of the microspores were performed by aceto carmine staining on squashes of anthers and observed under microscope (Figure 2A). In culture for androgenesis induction were placed only the anthers that contained microspores in or just before the first pollinic division (Figure 2B). The induction of somatic embryogenesis from anthers of the cultivars under study was performed with application of temperature treatment on Cp medium. After incubation of 12 days, the anthers were transfer to R_1 medium at +25±2°C, 12 hours light / 12 hours dark (Figures 2C and 2D). The number of callused and embryogenic anthers and number of embryos per 100 anthers were recorded. Embryos emerging from the anthers were transferred in test tubes onto V_3 medium for further development into plantlets (Figure 2E). Well-developed plantlets were planted into pots on and were placed in acclimatization chamber (Figure 2F).

Data analysis

All data of capsaicin content in pepper fruits, the percentage of callused anthers, the percentage of embryogenic anthers and the number of embryos per 100 anthers were statistically analysed using SPSS 10.0.5 and means were evaluated at the $p<0.05$ level of significance using Duncan's multiple range test. The Pearson's correlation coefficient between capsaicin content in pepper fruits and the percentage of callused anthers, the percentage of embryogenic anthers and the number of embryos per 100 anthers was performed in SPSS 10.0.5 program.

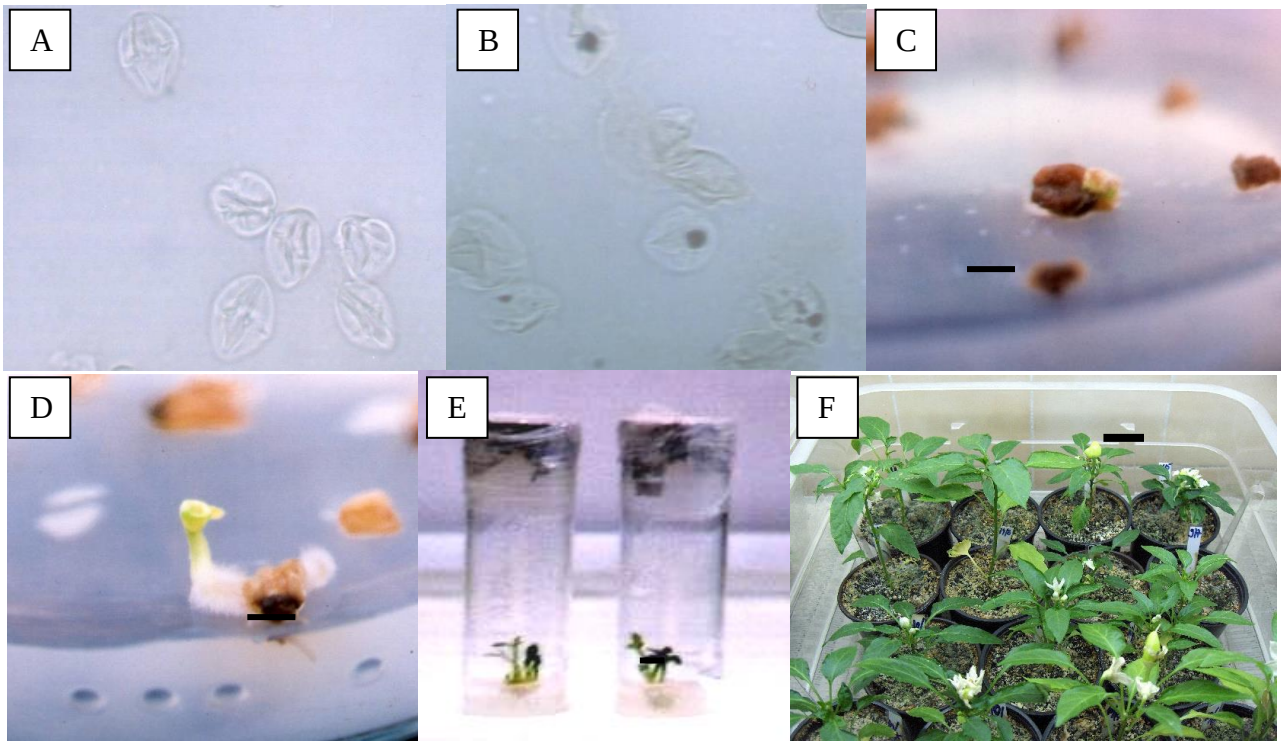

Figure 2. (A) Microspores in stadium of first pollinic mitosis before onset in the culture, acetocarmine squash method (x400); (B) Microspores after 6 days in culture, acetocarmine squash method (x400); (C) Direct embryo emerging from the anther after 30 days of culture; (D) Embryo emerging from the anther; (E) Plantlets on V_3 medium showing leaves; (F) Fully developed plants on acclimatization in climate chamber.
Scale bars: C and D 2.5 mm; E 1 cm; F 4 cm.

3. Results

The content of capsaicin in ethanol extract (oleorasin)

The content of capsaicin of fruits for each of the pepper cultivars is given in Table 1. As expected the pungent cultivars Feferona, Slatko Luta and Vezena Luta gave the higher capsaicin content as compared to the sweet cultivars (Sivrija, Golden Medal, Kurtovska Kapija), the bell-shape (Californian Wonder, Féherözön) and tomato-shape cultivar (Rotund). The capsaicin content of Feferona fruits is 4.5 folds higher as compared to the sweetest one Féherözön. The variety Sivrija, which contains 532.45 $\mu g \cdot g^{-1}$ capsaicin on fresh weight, according to the classification of the world pungency evaluators of different pepper species is on the boundary between the sweet and the moderate pungent fruits.

Table 1. The dependence of embryogenic response of cultivated pepper anthers on capsaicin content in pepper fruits (*Capsicum annum* L.)

Cultivars	Content of capsaicin in dry matter (µg/g)	Embryogenic response
Feferona (long, hot type)	899.76±51.80 a	No
Slatko Luta (long, moderate hot type)	863.30±3.88 ab	Poor
Vezena Luta (long, hot type)	618.65±1.90 bc	No
Sivrija (long, sweet type)	532.45±34.58 cd	No
Golden Medal (sweet spice type)	324.27±70.14 de	Poor
Kurtovska Kapija (sweet spice type)	271.11±5.04 e	Poor
Californian Wonder (bell-shaped, sweet type)	234.98±10.30 e	Fair
Feherözön (wax-bell-shaped, sweet type)	205.76±93.69 e	Excellent
Rotund (tomato shaped, sweet type)	216.86±9.39 e	No

Mean separation in columns by Duncan's multiple range test. In each column, values followed by the same letter do not differ significantly at $P \leq 0.05$.

Estimation of the androgenic potential in pepper anther culture

When anthers of the pepper cultivars were cultured on Cp medium under thermal treatment conditions, they responded with callus formation and/or direct embryogenesis (Table 2). The anthers that responded with direct embryogenesis did not form callus. On the contrary, the formed callus was not regenerative and not capable for further embryogenesis.

Table 2. Somatic embryogenesis in pepper anther culture (*Capsicum annum* L.)

Genotypes	Callused anthers (%)	Embryogenic anthers (%)	Number of embryos per 100 anthers
Feferona (long, hot type)	-	-	-
Slatko Luta (long, moderate hot type)	6.83 ± 0.75 c	2.43 ± 0.20 c	3.33 ± 0.57 c
Vezena Luta (long, hot type)	26.51 ± 7.85 a	-	-
Sivrija (long, sweet type)	14.23 ± 1.85 b	-	-
Golden Medal (sweet spice type)	3.33 ± 1.29 c	3.31 ± 0.24 c	3.66 ± 0.57 c
Kurtovska Kapija (sweet spice type)	8.25 ± 0.44 c	1.55 ± 0.50 c	2.66 ± 0.57 d
Californian Wonder (bell-shaped, sweet type)	15.12 ± 5.00 b	6.66 ± 0.28 b	5.66 ± 0.57 b
Feherözön (wax-bell-shaped, sweet type)	3.92 ± 1.38 cd	31.09 ± 6.02 a	35.00 ± 1.00 a
Rotund (tomato shaped, sweet type)	19.00 ± 1.00 b	-	-

Mean separation in columns by Duncan's multiple range test. In each column, values followed by the same letter do not differ significantly at $P \leq 0.05$.

The only cultivar that did not respond to the applied conditions at all was Feferona (Table 2). The cultured anthers of Slatko Luta, Golden Medal, Kurtovska Kapija, California Wonder and Féherözön responded with callus formation and direct embryo formation, while the cultured anthers of Vezena Luta, Sivrija and Rotund responded with callus formation (Table 2). The androgenic potential was determined on the basis of number of embryos from responding anthers and anthers that gave embryos (Table 1, Table 2). The highest frequencies of responsive anthers (31.09 %) as well as the greatest number of emerging embryos were obtained from Hungarian cultivar Féherözön. Fair response showed only the cultivar California Wonder, while poor frequency of androgenesis occurred in 3 cultivars: Slatko Luta, Golden Medal and Kurtovska Kapija. Among the nine cultivars tested Feferona, Vezena Luta, Sivrija and Rotund were considered to be non-androgenic.

The mechanism of cold and hot thermal shock in induction of somatic embryogenesis is explored and discussed by many authors [32,41-43]. According to the literature, the hot thermal stress (+35^{o}C) has greater effect than the cold one (+7^{o}C) in the process of stimulation of microspores division [44,45]. Another authors used different stimulations and different chemical agents as thidiazuron [46,47] and charcoal [31]. Although different pre-treatments were applied, in the most cases the hot varieties possess weaker reaction or do not form embryos at all. Mitykó and Fari [48] concluded that bell-shape varieties have the highest androgenic ability, while the rest showed very low or no androgenetic activity.

This was confirmed with the results of our research work, where bell-shape varieties were embriogenicaly more potential as compared to the hot and the sweet ones, which is in agreement with the conclusions of the known research workers. The hottest variety Feferona (899.76 mg·g^{-1} capsaicin) did not react on CP medium. The anthers neither formed callus embryos, while the anthers of Féherözön variety (205.76 mg·g^{-1} capsaicin) showed low callus formation (3.92%), but the androgenic ability was the highest on the same medium with 31.09% embryogenic anthers (Table 1, Table 2)

Correlation between capsaicin content and androgenic processes

The Pearson's correlation coefficient (r) showed that all androgenesis parameters are in negative correlation with the capsaicin content of the pepper fruits. The strongest negative correlation was determined between the capsaicin content and percentage of embryogenic anthers (r = -0.418). The correlation coefficient between the capsaicin content and the number of embryos per 100 anthers is r = -0.389, while between the capsaicin content and percentage of callused anthers is r = -0.213 which is the weakest correlation.

Our results showed that the pungent pepper cultivars possess either poor androgenic potential or can be considered as non-androgenic cultivars, while the sweet and bell-shaped varieties response with embryo formation.

The pepper pungency is connected with the capsaicin content, which is synthesising also *in vitro* conditions and we can conclude that the capsaicin is an inhibitor of embryogenesis in pepper anthers culture.

.

4. Discussions and Conclusion

The inhibitory activity of the capsaicin has influence on the haploid embryos formation. For example, the varieties with higher capsaicin content do not show androgenic response. The only exception from all the cultivars under investigation is tomato shaped Rotund which beside the low concentration of capsaicin did not show androgenic potential. Most probably it is matter or low genetic predisposition of the cultivar for androgenesis *in vitro* conditions. All the rest of the cultivars showed dependence between the capsaicin content and androgenic potential in *in vitro* conditions. The mechanism of capsaicin influence on the *in vitro* conditions processes is still unknown. Further research work for clarifying of the inhibitory effect of endogenous capsaicin in the tissue and organs culture of the *Capsicum* genus is required.

On the other hand, the comparison of our results with these of Mitykó and Fari [29] in the pepper anther culture of the variety Californian Wonder 14.6% of the anthers formed embryos, while 48.3% of the anthers of Féherözön variety were embryo productive, which gave lower percentage. In our research, the Californian Wonder gave 6.16%, while Féherözön 33.66% androgenetically active anthers. But, according to the classification of the aforementioned authors, in the both experiments the results are identical, where the variety Californian Wonder had average, whereas the variety Féherözön had excellent androgenic potential. The pepper androgenesis is very restrictive phenomenon. The number of embryos formed from anthers is very low. In order this phenomenon to be stimulated in the literature many different treatments are mentioned (hot and cold shock treatment, treatment with colchicine), but even when the same simulative treatment are applied to hot and sweet varieties, the hot varieties regarding the androgenic response are evaluated as low - responsive and not - responsive genotypes. Embryo induction rates of pepper anther culture in nine cultivars under study is very low, from 1.55% embryogenic anthers in Kurtovska Kapija to 31.09% embryogenic anthers in Féherözön. The hottest cultivar Feferona in anther culture did not react at all either with callus formation or embryogenic. The anthers on R1 medium, after the incubation period on CP medium, were necrotic i.e. are swollen by the medium. The hot cultivar Vezena Luta and the sweet cultivar Sivirija did not formed embryos at all, but the callus formation is major phenomenon.

If the correlation between the capsaicin content and the level of androgenesis is analyzed through the parameters % of callus formation, % of embryogenesis and number of formed embryos per 100 anthers there is justification of the opinion that there is inhibition as result of the secondary metabolites especially the capsaicin as leading alkaloid in *in vitro* pepper anther culture.

Acknowledgements

The results of this paper are part of the implementation of the bilateral MK-BG scientific project „Obtaining haploid from pepper anther culture (*Capsicum annuum* L.) and their involvement in the selection process". The process of androgenesis is derived at the Laboratory of plant biotechnology, Department of Plant Production, Faculty of Agriculture, University Goce Delchev – Stip, R. Macedonia. Capsaicin detection is performed at the Faculty of Technical and Metallurgy, Department of Food Technology, University Ss. Cyril and Methodius – Skopje, R. Macedonia. The authors are thankful for established cooperation and the opportunity to realize the determination of the content of capsaicin in different cultivars of peppers.

References

[1] Basu, K.S. and A. De Krishna (eds). 2003. *Capsicum*. Medicinal and aromatic Plants – *Industrial Profiles*. London: Taylor & Francis.

[2] Suzuku, T., H. Fujiwake, and K. Iwai. (1980). Intracellular localization of capsaicin and its analogues, capsaiconoides, in *Capsicum* fruit 1. Microscopic investigation of the structure of the placenta of *Capsicum annum* var. *annum* cv. Karayatsubusa. *Plant Cell Physiol*. **21**: 839-853.

[3] Bennett, D.J. and G.W. Kirby. 1968. Constitution and biosynthesis of capsaicin. *J. Chem. Soc.* **C**: 442-446.

[4] Walsh, B.M. and S.B. Hoot. 2001. Phylogenic relationships of *Capsicum* (Solanaceae) using DNA sequences from two non-coding regions: the chloroplast *atpB-rbcL* spacer region and nuclear waxy introns. *Int. J. Planta Sci.* **162**: 1409-1918.

[5] Govindarajan, V.S. 1986. *Capsicum*- production, technology, chemistry and quality - Part III. Chemistry of the colour, aroma and pungency stimuli, *CRC, Critical Review in Food Science and Nutrition* **24**(3): 254-355.

[6] Tod, J. and N. Catt. 2002. Red "why are they so hot" chili peppers, Keble College, Oxford.

[7] Leete, E. and M.C.L. Louden. 1968. Biosynthesis of capsaicin and dihydrocapsaicin in *Capsicum frutescens*. *J. Am. Chem. Soc*. **90**: 6837-6841.

[8] Koleva-Gudeva, L. 2003. The effect of incubation treatment on the pepper (*Capsicum annuum* L.) androgenesis, *Yearbook of Institute of Southern Crops – Strumica.* **3**: 87-94.

[9] Koleva-Gudeva, L., V. Rafajlovska, and M. Spasenoski. 2004. *In vivo* and *in vitro* content of capsaicin in pepper (*Capsicum annuum* L.). VIII Symposium Biotechnology and Agroindustry, Velika Plana, Serbia. *Proceeding of symposium* pp. 252-259.

[10] Sasson, A. 1991. Production of useful biochemicals by higher-plant cell cultures: biotechnological and economic aspects. *CHIEAM, Options Mediterraneennes* **14**: 59-74.

[11] De Witt, D. 1999. The nature of capsaicin: The Chile Pepper Encyclopaedia, Morrow Cookboks, NY, USA.

[12] Bernstein, JE. 1991. Method and composition utilizing capsaicin external analgetic - with anesthetic desensitize. Galenpharma Inc. US 4997853.

[13] Bunk, S. 2000. Cloning the Capsaicin Receptor. *The Scientist* **14**(2): 19.

[14] Cheng, C.L., J.C. Liu, S.Y. Chang, C.P. Ma, and W.C. De Groat. 1999. Effect of capsaicin on the micturition reflex in normal and chronic spinal cord-injured cats. *Am. J. Physiol* **277** R7:86-94.

[15] Davison, M.W. 2000. Capsaicin – Molecular Expressions: Phytochemical Gallery National High Magnetic Field Laboratory, Florida State University, USA.

[16] Holt, S.D. 1999. Pain reliever and method of use – capsaicin entrant analgesic. *Medical Merchandising Inc,* US 5856361.

[17] Košťálová, D. 2002. Topical capsaicin in dermatological and peripheral pain disorders, Katedra farmakgnózie a botaniky Farmaceutická fakulteta UK, Bratislava.

[18] Tominaga, M. 1998. The cloned capsaicin receptor integrates multiple pain product stimuli. *Neuron* **21**(3): 644-645.

[19] Ying-Yue, W, H. Chi-Tzong, C. Wen-Ta, and F. Jia-You. 2001. I*n vitro* and *in vivo* evaluations of topically applied capsaicin and nonivamide from hydrogels. *Int J of Pharmaceutics* **224**: 89-104.

[20] Kurita, S. 2002. Studies on the antimicrobial mechanisms of capsaicin using yeast DNA microarray. *Biosci Biotechnol Biochem* **66**(3): 532-536.

[21] Nitin Pal Kalia, Priya Mahajan, Rukmankesh Mehra, Amit Nargotra, Jai Parkash Sharma, Surrinder Koul and Inshad Ali Khan. 2012. Capsaicin, a novel inhibitor of the NorA efflux pump, reduces the intracellular invasion of *Staphylococcus aureus. Journal of Antimicrobial Chemotherapy* **67**(1**0**): 2401-2408

[22] Sinha, R. K., Hahn, G., Singh, P.K., Suhane, R. K., and Anthonyreddy, A. 2011. Organic Farming by Vermiculture: Producing Safe, Nutritive and Protective Foods by Earthworms, *American Journal of Experimental Agriculture* **1**(4): 363-399.

[23] Ubulom1, P.M.E.N.G., Imandeh, C.E., Udobi and Ilya, I. 2012. Larvicidal and Antifungal Properties of *Picralima nitida* (Apocynaceae) *Leaf Extracts, European Journal of Medicinal Plants* **2**(2): 132-139.

[24] Mori A,Lehmann S,O'Kelly J,Kumagai T,Desmond JC,Pervan M,McBride WH,Kizaki M,Koeffler HP.Cancer 2006. Capsaicin, a component of red peppers, inhibits the growth of androgen-independent, p53 mutant prostate cancer cells. *Cancer Res*. **66**(6): 3222-3229.

[25] Archer, E.V. and D.W Jones. 2002. Capsaicin pepper, cancer and ethnicity. *Medical Hypotheses* **59**(4): 450-457.

[26] Dasgupta, P., V. Chandiramani, M.C. Parkinson, A. Beckett, and C.J. Fowler. 1998. Treating the human bladder with capsaicin: Is it safe?. *Euro Urol* **33**(1): 28-31.

[27] Surh, J. 2002. More than pain: capsaicin in hot chili peppers makes tumour cells commit suicide. *J Nat Cancer Inst.* **94**(17): 1263-1265.

[28] Zhang, J., M. Nagasaki, Y. Tanaka, and S. Morikawa. 2003. Capsaicin inhibits growth of adult T-cell leukemia cells. *Leukemia Research* **27**(3): 275-283.

[29] Mitykó, J., A. Andrasfalvy, G. Csillery, and M. Fary. 1995. Anther-culture in different genotypes and F_1 hybrids of pepper (*Capsicum annuum* L.). *Plant Breed*. **114**: 78-80.

[30] Rodeva, V.N., T.P. Irikova, and V.J. Todorova. 2004. Anther culture of pepper (*Capsicum annuum* L.): Comparative study of effects of the genotype. *Biotechnol & Bitechnol Eq* **18**(3): 34-38.

[31] Özkum Çiner, D. and R. Tripirdamaz. 2002. The effects of cold treatment and charcoal on in vitro androgenesis of pepper (*Capsicum annuum* L.). *Turk J Bot* **26**: 131-139.

[32] Dolcet-Sanjuan, R., C. Claveria, and A. Huerta. 1997. Androgenesis in *Capsicum annuum* L. - Effects of Carbohydrate and Carbon Dioxide *Enrichments. J Amer Soc Hort Sci* **122**(4): 468-475.

[33] Irikova, T. and V. Rodeva. 2004. Anther culture of pepper (*Capsicum annuum* L.): the effects of nutrient media. *Capsicum and Eggplant Newsletter* **23**: 101-104.

[34] Koleva-Gudeva, L. and M. Spasenoski. 2001. The effect of some citokinines on pepper organogenesis (*Capsicum annuum* L. cv. Kurtovska kapija and Zlaten medal) cultured *in vitro*. *Yearbook of the Institute of Southern Crops – Strumica* **1**: 31-35.

[35] Mitykó, J. and M. Fary.1997. Problems and results of doubled hapliod plant production in pepper (*Capsicum annuum* L.) via anther and microscope culture. Hort Biotech *In Vitro* Cult Breeding. *Acta Hort* **447**: 281-287.

[36] Quin, X. and G.L. Rotino. 1993. Anther culture of several sweet and hot pepper genotypes. *Capsicum and Eggplant Newsletter* **12**: 59-62.

[37] Ltifi, A. and G. Wenzel. 1994. Anther culture of hot and sweet pepper (*Capsicum annuum* L.): Influence of genotype and plant growth temperature. *Capsicum and Eggplant Newsletter* **13**: 74-77

[38] Wang, J.J., C.S. Sun, C.C. Wang, and N.F. Chein. 1973. The induction of pollen plantlets of *Triticale* and *Capsicum annuum* from anther culture. *Sci Sinica* **16**(1): 147-151.

[39] George, L. and S. Narayanaswamy. 1973. Haploid *Capsicum* through experimental androgenesis. *Protoplasma* **78**: 467-470.

[40] Kuo, J.S., Z.Z. Wang, N.F. Chien, S.J. Ku, M.L. Kung, H.C. Hsu. 1973. Investigation of the anther culture *in vitro* of *Nicotiana* and *Capsicum annuum* L.. *Acta Bot Sin* **15**(1): 43-47.

[41] Dumas de Valux, R., D. Chambbonet, and E. Pochard. 1981. Culture *in vitro* d`anterès de piment (*Capsicum annuum* L.): amèlioration des taux d`obtention de

plantes chez diffèrents gènotypes par des traitements à +35ºC. *Agronomie* **1**(10): 859-864.

[42] Matsubara, S., M. Yamamoto, J. Man Hyun, K. Murakamy, and H.J. Man. 1998. Embryoid and callus formation from microspores by anther culture from July to November in pepper (*C. annuum* L.). *Sci. Rep Fac of Agriculture, Okayama University* **87**: 117-122.

[43] Munyon, I.P., J.F. Hubstenberger, and G.C. Phillips. 1989. Oring of plantlets and callus obtained from chilli pepper anther cultures. *In Vitro Cell Dev Biol* **25P**: 293-296.

[44] Bárány, I., P. González-Melendi, B. Fadón, J. Mitykó, and C. Maria Risueño. 2005. Microspore-derived embryogenesis in pepper (*Capsicum annuum* L.): subcellular rearrangements through development. *Biol Cell* **97**: 709-722.

[45] Kim, M., J. Kim, M. Yoon, D. Choi, and K. Lee. 2004. Origin of multucellular pollen and pollen embryos in cultured anthers of pepper (*Capsicum annuum*). *Plant Cell, Tissue and Organ Culture* **77**: 63-72.

[46] Binzel, M., N. Sankhla, Joshi S, and D. Sankal. 1996. Induction of direct somatic embryogenesis and plant regeneration in pepper (*Capsicum annuum* L.). *Plant Cell Reports* **15**: 536-540.

[47] Peddaboina, V., C. Thamidala, and S. Karampuri. 2006. *In vitro* shoot multiplication and plant regeneration in four *Capsicum* species using thidiazuron. *Scientia Horticulturae* **107**: 117-122.

[48] Mitykó, J., L. Szabó, and B. Barnabás. 1999. Cholchicine induced ultrastructural changes in barley and pepper microspores. *J Slovak Acad Sci* **54**: 24-25.