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OBTAINING HAPLOIDS IN ANTHER CULTURE OF PEPPER *Capsicum annuum* L. AND THEIR INCLUSION IN THE BREEDING PROCESS

Velichka Rodeva*§, Liljana Koleva-Gudeva§, Stanislava Grozeva*, Fidanka Trajkova****

Abstract

The frequency of obtained androgenic plants depends highly on the genotype; therefore the low rate of haploid recovery limits the utility of anther culture in pepper breeding.

The aim of this study was establishment of effective *in vitro* technology for study of haploid and diploid plant regenerants; induction of embryogenesis in pepper anther culture; development of the embryos into regenerants as well as successful adaptation and acclimatization of regenerants from sterile to greenhouse conditions. In the present study, the effectiveness of induced androgenesis in anther culture of several Bulgarian and Macedonian pepper genotypes was investigated.

The collected seed material is excellent possibility for further breeding processes, cytogenetic and other molecular level research.

The results of this paper derived from international bilateral Macedonian-Bulgarian Joint Research Project: "Obtaining haploids in anther culture of pepper *Capsicum annuum* L. and their inclusion in the breeding process", managed by the first two authors and with participation of the coauthors.

Key words: *in vitro embryogenesis*, *Capsicum annuum* L., *genotype*

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ДОБИВАЊЕ НА ХАПЛОИДИ ВО КУЛТУРА НА АНТЕРИ ОД ПИПЕРКА *Capsicum annuum* L. И НИВНО ВКЛУЧУВАЊЕ ВО ПРОЦЕСОТ НА СЕЛЕКЦИЈА

Велика Родева*, Лилјана Колева-Гудева, Станислава Грозева*, Фиданка Трајкова****

Краток извадок

Зачестеното добивање андрогенетски растенија е многу зависно од генотипот, па малата вредност на хаплоидното обновување го ограничува користењето на културата на антери во селекцијата на пиперка.

Целта на ова истражување беше воспоставување на ефективна *in vitro* технологија за проучување на хаплоидни и диплоидни растителни регенеранти; индукција на ембриогенеза во култура на антери од пиперка; развој на ембриони во регенеранти, како и успешна адаптација и аклиматизација на регенерантите од стерилни во оранжериски услови.

Колекцинираниот семенски материјал дава одлична можност за понатамошни процеси на селекција, цитогенетски и други истражувања на молекуларно ниво.

Резултатите презентирани во овој труд се произлезени од интернационалниот билатерален македонско-бугарски истражувачки проект за соработка „Добивање на хаплоиди во култура на антери од пиперка *Capsicum annuum* L. и нивно вклучување во процесот на селекција" воден од првите двајца автори со учество на коавторите.

Клучни зборови: *in vitro ембриогенеза, Capsicum annuum* L., *генотип*

1. Introduction

Pepper is one of the most widespread vegetable crops, economically important for countries of the Balkan region including Macedonia and Bulgaria. The specific genetic diversity of local forms here is unknown for many countries in the world.

Creation of haploids and spontaneous doubled haploids in anther culture is a method applied in pepper plant genetics and breeding because of the importance of the haploids in the study of the gene map (Yoo et al., 2003), for genetic manipulations, molecular investigations and development of disease and stress resistant lines (Gyulai et al. 2000; Ochoa-Alejo and Ramirez-Malagon, 2001; Arendo Andres et al., 2004). Wang et al. (1973) obtained the first haploid pepper in anther culture. Haploid morphogenesis of *Capsicum* species was studied by George and Narayanaswamy (1973) and Kuo et al. (1973) although the production of haploid plants was very low.

The first successful reproductive method for production of pepper haploids was developed by Dumas de Valux et al. (1981). The research on androgenesis was intensive during the last years of the twentieth century, but the regenerants were a mixture of haploid and diploid plants. In order to increase the effectiveness of somatic embryogenesis and haploid production different stress treatments were used (Mityko et al., 1995; Mityko and Fari, 1997; Supena et al., 2006).

The induction of somatic embryogenesis in culture of anthers when microspores are in the stage of first pollen division (n=x) is successful for obtaining haploid and diploid regenerants (Koleva-Gudeva, 2003). Nowadays, androgenesis under *in vitro* conditions is effective method for induction of haploids (Koleva-Gudeva et al., 2007).

This work is aimed to study *in vitro* embryogenic answer of anthers and plant regeneration in local Balkan region pepper lines, varieties and F_1 hybrids in different cultivation media.

2. Materials and Methods

2.1. Experiments carried out in Macedonia

Nineteen pepper genotypes were used as anther-donor plants. Anther-donor plants were grown under greenhouse conditions. Donor plants were used during the four weeks after the first flower buds had appeared. The flower buds were harvested when the corolla was of the same length as the calyx or slightly longer.

The developmental stage of the macrospores was determined in microscopic slides of acetocarmine squashes. Flower buds were surface sterilized in 70% ethanol for several seconds, then in 5% Ca $(ClO)_2$ + 2-3 drops Tween 20 for 10 minutes, and rinsed three times in sterile distilled water. After the removal of the filaments, anthers from three flower buds were placed in Petri dish (6 cm diameter), with the concave face down, touching the culture medium.

The method of Dumas de Valux et al. (1981) was used for androgenic induction. According to the method, the anthers were cultivated on CP medium + 0,01mg·l^{-1} KIN + 0,01 mg·l^{-1} 2,4-D with incubation of 8 days in darkness at 35±2ºC, the following 4 days the anthers were transferred to clime chamber at 25±2ºC with photoperiodism 12h light/12h dark. Afterwards, the anthers were subcultured on R_1 medium + 0,01 mg·l^{-1} KIN and placed in clime chamber at 25±2ºC with photoperiodism 12h light/12h dark. Young shoots emerging from the anthers were transferred onto hormone free V_3 media in order roots to be formed.

2.2. Experiments carried out in Bulgaria

Donor plants from 22 lines, cultivars and F_1 hybrids grown under greenhouse conditions were used for collecting of flower buds during the period from May to October. Anthers 3-4,5 mm in size were placed on a medium containing micro- and macrosalts by Murashige and Skoog (1962), vitamins by Gamborg et al., (1968), 0,3 mg l^{-1} 2,4-D, 0,1 mgl^{-1} Kinetin, 0,005 mg l^{-1} Biotin, 0,1mg l^{-1} Glycine, 0,04mgl^{-1} Vitamin B_{12}, 30 g l^{-1} Sucrose and 0,7 % Agar. The cultivated anthers were treated in darkness with 35±1°C for the first 8 days and later were incubated on the same medium without growth regulators in the condition of growth chamber at 26ºC ± 1ºC, 4000 lux under 16/8 h day/night.

The plantlets were planted on sterile mixture of perlite: peat : sand (1:1:1) and acclimatized in clime chamber, and afterwards placed in greenhouse under cover in order crosspollination to be barred.

3. Results and Discussion

3.1. Results obtained in Macedonia

Not all genotypes under investigation were able to produce haploid embryos. After the induction period on CP medium for 12 days the anthers were subcultured on R_1 medium, where since the beginning the embryos showed totipotency, progression in development, growth and shoot formation.

The shoots continued the development on V_3 medium, where in absence of phytohormones young plants were formed (Figure 1a). The rooting was also on V_3 medium and well rooted shoots were transferred on sterile mixture of sand : perlite : peat in ration 1:1:1 (Figure 1b). In this stage the plants were ready for adaptation and acclimatization in greenhouse conditions.

From 19 pepper genotypes under investigation, 12 possessed potential for formation of direct somatic embryos. The hot genotypes (with exception of *Rotund, Kurtovska kapija TU* and *Kurtovska kapija MK)* did not show androgenic potential, i.e. in anther culture did not form haploid shoots.

According to the classification of Mityko and Fari (1997) for identification of androgenic potential according to the percentage of anthers that give embryos, pepper types are classified into:

- poor androgenic potential - less than 5% embryogenic anthers
- fair androgenic potential - 5.1 - 15% embryogenic anthers
- good androgenic potential - 15.1 - 30% embryogenic anthers
- excellent androgenic potential - over 30% embryogenic anthers

The results from our research showed that somatic embryos are formed on CP medium with heat temperature stress (+35°C) which is in concord with the findings of Dumas de Valux et al. (1981).

From all 19 genotypes, 12 showed ability for embryo formation (Table 1):

- 2 genotypes with good androgenic potential: *Tura* and *Féherözön;*
- 4 gentypes with fair androgenic potential: *Pritavit F1*, *Californian wonder*, *Zlaten medal SR* and *Majori;*
- 6 genotypes with poor androgenic potential: Piran, Zlaten medal ЈЊТ, Tomato shaped sweet, *Kurtovska kapija BG, Kurtovska kapija SR* and *Slatko luta*;
- 7 genotypes do not possess androgenic potential: *Feferona, Vezena luta, Sivrija, Rotund, Kurtovska kapija TU, Kurtovska kapija MK* and *Bonbona*.

Seed material was collected from four genotypes: Kurtovska kapija SR, *Zlaten medal SR, Piran* and *Féherözön* .The collected seed material is good base for further cytogenetic and molecular research and involvement in process of pepper breeding in order better varieties to be created.

3.2. Results obtained in Bulgaria

A very important factor with influences the success of the *in vitro* anther culture is careful selection of the appropriate late uninucleate development stage of microspores. In the result of our experiments it was established that at this stage different length of the corolla petals and the calyx sepals occurs and mostly is between 3,0 mm to 4,5 mm for the corolla and 4,0 mm to 5,3 mm for the calyx in the studied genotypes. We registered microspores in different stage of microsporogenesis in the anthers with the same size which proves the importance of preliminary morphological and cytological characterization in choice of anthers for *in vitro* cultivation (Ozkum and Tipirdamaz, 2002). In the most of the studied genotypes it was established correlation between the stage "uninucleate pollen", suitable for androgenesis and appearance of light anthocyan color on the anther tips with exception of non-anthocyan anthers of *line P295/03* and *line 295/00* F_3, with coming of the anthocyan color in the late stage of bud development.(Fig. 2). In contrast with the opinion of some authors, buds with the length of corolla petals equal to the length of calyx sepals include microspores at the late uninucleate stage, in the studied Bulgarian genotypes this stage was observed in buds with slightly longer corolla petals.

In result of the experimental work it was established induction of embryogenesis in five from all 22 studied pepper genotypes. The answer of these five genotypes is presented in Table 2. Data in Table 2 show that from 1 315 cultivated anthers embryogenic reaction is registered in 1,67% and from 8,90% obtained embryogenic structures 1,82 plants per 100 anthers is regenerated. The highest percentage of embryogenic anthers is registered in variety Strjama (7,60%) and line 2087/01 (2,14%). The lowest value of this characteristic is observed in variety *Bel Rubi* and *line1647* (0,32%). Development of the obtained embryoids to plant-regenerants is not observed in the both genotypes. The

highest number of developed regenerants per 100 anthers (12,12) is established in variety *Strjama* in correlation with the highest number of obtained embryoid structures per 100 anthers (56,1). Comparatively high number of formed embryos is registered in anthers of line 2087/01 and variety *Zlaten medal* (8,02 and 4,79 per 100 anthers respectively), but considerable lower number of the structures are developed to plant-regenerants (0,53 and 1,34 per 100 anthers respectively) (Fig. 3). The established differences in the embryogenic answer of the studied genotypes probably are due to the cultivation conditions and to the specific genotype characteristics – more or less predetermined for embryogenesis. According some authors only some of the pepper genotypes have a capacity for embryogenic development. Qin and Rotino (1993) and Mityko et al. (1995) report about sporadic embryogenesis in different pepper genotypes. Mityko and Fary (1997), established better embryogenic answer in sweet pepper genotypes than the spice pepper genotypes.

The development of the obtained embryogenic structures to the plant-regenerants was very slow depending from the specificity of the genotype, the season of obtaining and cultivation conditions (Fig. 4). It was very difficult to micropropagate the developed plant-regenerants – a problem existing in the most working on pepper laboratories in the world. The higher phenolic contents in plant tissue make difficult *in vitro* pepper cultivation what is the reason for carrying out of extensive research for optimal media determining, antioxidant explant treatment and the period of the subculturing (Zhenjiu and Wang, 1990).

Seeds from 6 plants are collected from all 12 adapted plants which prove their diploid nature. Six of the regenerants are sterile, weak and slow developing plants, probably due to their haploid nature.

4. Conclusions

Pepper is recalcitrant in cultures *in vitro* and the results in cell and tissues cultures are moderate. Anther culture is the only exception from this rule (Mityko and Fari, 1997).

The results regarding the process of embryo formation on different media under different thermal conditions showed that the formation of haploid embryos occurred only in the CP medium exposed to heat-thermal stress (+35^{o}C), what is in accordance with the findings of De Valux (1981). However, Irikova and Rodeva (2004) reported no embryos formation for the same medium and cultivation conditions.

There were established differences in the anther embryogenic capacity and development of the obtained plant-regenerants depending on the genotype. Embryogenic answer and regeneration in the optimized medium of Murashige and Skoog /1962/ were registered only in 5 specific for Balkan region local

pepper lines, varieties and F_1 hybrids from all 22 studied genotypes. Haploid and diploid plants are obtained and grown *in vivo* and seeds are collected for future experiments.

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Tab. 1 Haploid embryo induction from anthers of different pepper genotypes
Таб. 1 Индукција на ембриоиди од антери на различни генотипови пиперка

Pepper genotype Генотип на пиперка	Total number of anthers Вкупен број антери	Embryogenic anthers (%) Ембрио-генетски антери (%)	Number of embryos per 100 anthers Број на ембриоиди на 100 антери	Embryo-genic response Ембрио-генетски одговор
Féherözön *фехерозон*	1502	17.39 a	32.60 bc	Good Добар
Tura *тура*	300	17.05 a	17.05 ab	Good Добар
Pritavit F1 *притавит F1*	330	9.23 abc	9.39 abc	Fair Доволен
California wonder *калифорниско чудо*	151	6.67 abc	5.67 c	Fair Доволен
Zlaten medal SR *златен медал СР*	1031	6.12 abc	8.97 bc	Fair Доволен
Majori *мајори*	330	5.83 abc	6.73 c	Fair Доволен
Piran *пиран*	823	5.03 abc	34.05 ab	Poor Слаб
Zlaten medal ŠT *златен медал ШТ*	723	4.29 bc	18.57 bc	Poor Слаб
Tomato shaped sweet *доматовидна блага*	360	4.17 bc	4.54 c	Poor Слаб
Kurtovska kapija BG *куртовска капија БГ*	620	2.90 bc	50.55 a	Poor Слаб
Kurtovska kapija SR *куртовска капија СР*	875	2.73 bc	10.20 bc	Poor Слаб
Slatko luta *слатко лута*	140	2.43 bc	3.33 c	Poor Слаб
Feferona *феферона*	79	0.00 c	0.00 c	No Нема
Vezena luta *везена лута*	83	0.00 c	0.00 c	No Нема

Sivrija *сиврија*	104	0.00 c	0.00 c	No Нема
Rotund *ротунд*	109	0.00 c	0.00 c	No Нема
Kurtovska kapija TU *куртовска капија ТУ*	236	0.00 c	0.00 c	No Нема
Kurtovska kapija MK *куртовска капија МК*	122	0.00 c	0.00 c	No Нема
Bonbona *бонбона*	270	0.00 c	0.00 c	No Нема

Mean within a column followed by the same letters are not significantly different at $p < 0.05$ according to Duncan's multiple range test.

Tab. 2 Embryo induction and plant regeneration in anther culture of different pepper genotypes

Таб. 2 Индукција на ембриоиди и регенерација на растенија во култура на антери од различни генотипови пиперка

Pepper genotype Генотип на пиперка	Total number of anthers Вкупен број на антери	Embryo-genic anthers (%) Ембрио-генетски антери (%)	Number of embryos per 100 anthers Број на ембриоиди на 100 антери	Plant-regenerants per 100 anthers Растенија-регенеранти на 100 антери	Embryogenic Response Ембрио-генетски одговор
Zlaten medal *златен медал*	522	1.14 c	4.79 c	1.34	Poor
Bel Rubi *бел руби*	312	0.32 d	0.64 d	0.00	Poor
Strjama *стријама*	132	7.60 a	56.1 a	12.12	Fair
Line 1647 *линија 1647*	162	0.32 d	0.62 d	0.00	Poor
Line 2087/01 *линија 2087/01*	187	2.14 b	8.02 b	0.53	Poor
Average **Просек 1**	**1315**	**1.67**	**8.90**	**1.82**	

Mean within a column followed by the same letters are not significantly different at $p < 0.05$ according to Duncan's multiple range test.

Fig. 1 Morphological characteristics of pepper anther buds when microspores are in uninuclphase
Сл. 1 Морфолошки карактеристики на пупките од пиперка кога микроспорите се наоѓаат во фаза на идентични јадра

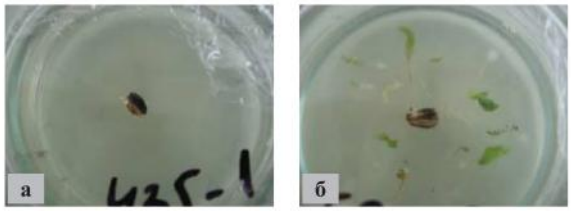

Fig. 2 Embryogenic reaction in pepper anther culture of a. *Line 2087/0*1 and b. variety *Strjma*
Сл. 2 Ембриогенетска реакција на култура на антери од пиперка на: а) *линија 2087/1* и б) сорта *стријама*

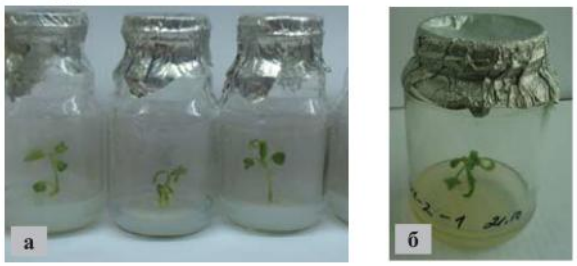

Fig. 3 a.b. Development of the embryos into regenerants on V3 medium
Сл. 3 a.b. Развивање на ембриоидите во регенеранти на V3 медиум

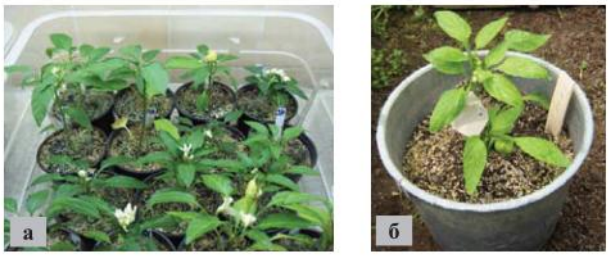

Fig. 4 a. Acclimatization of the regenerants in clime chamber under controlled conditions
b. Adaptation of the regenerants in greenhouse conditions
Сл. 4 a. Аклиматизација на регенерантите во клима комора во контролирани услови
b. Адаптација на регенерантите во оранжериски услови