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Effect of incubation period and culture medium on pepper anther culture

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Recurrent surge in demand of multipurpose peppers have positively encouraged pepper breeding community to study diverse aspects of pepper genetics, physiology, breeding and biotic stresses, however research on application of anther culture in pepper is still limited. Current study was aimed to assess the *in vitro* androgenic response of two prominent Bulgarian pepper varieties in five different duration times (12, 14, 16, 18 and 20 days) on incubation period and regeneration mediums containing kinetin (0.1, 0.2 and 0.3 mg L^{-1}) and combination of zeatin (0.25 mg L^{-1} 0.5 mg L^{-1}) and indole acetic acid (IAA) (0.1 mg L^{1}). Experimental results indicated specific requirements for composition of culture medium and duration time depending on genotype. Prolonged anthers incubation on induction medium had a positive effect on embryo formation and their conversion into plantlets compared to regeneration medium variants. Highest number of embryos and plant-regenerants of variety Stryama was achieved after 18 days incubation period and medium variant containing 0.1 mg L^{-1} kinetin. Embryo induction in variety Zlaten Medal 7 was higher after 16 days and control medium without plant growth regulators, while regeneration at 20 days also on control medium variant.

Keywords: *Capsicum annuum*, embryo regeneration, microspore embryogenesis, growth regulators

Introduction

Continuous interest and growing demand for peppers is persistently on the rise and has encouraged capsicum community to dissect in depth understanding of fruit morphology, biochemical profiling and tolerance to biotic and abiotic stresses[1-3]. Conventionally, classical breeding has been adopted to evaluate and breed peppers across different parts of the world[4-6] and lately use of high-throughput fruit phenotyping[7] and genotyping tools[8-9] have also adapted to improve the breeding efficiency. The *in vitro* embryogenesis techniques in pepper have been used since 1973[10-12] and have proven to be useful in reducing breeding cycle in comparison to classical breeding[13-15] however, their comprehensive understanding is still limited due to low efficacy and likelihood of somatic regenerants[16-17].

Microspore embryogenesis derived from anther culture is an important method to generate haploid and double-haploid embryos and plant-regenerants to accelerate the breeding process. Pure homozygous lines are developed in one-step procedure by microspore embryogenesis and a good source of diversity of forms with improved morphological traits, productivity and disease resistance[18-19]. Pepper (*Capsicum annuum* L.) is categorized as a recalcitrant plant species to *in vitro* manipulation and effectiveness of androgenesis is still low. Over the years, anther or microspore culture has evolved significantly[20] and has been successfully applied in different vegetable crops[21-22]. Multiple studies demonstrated that a high frequency of anther-derived embryos were able to be obtained, but few of them were able to develop into normal plants[23]. Grozeva *et al*[24] established embryo formation ranging from 0 to 9% and subsequently developed eight regenerants from these embryos and similar results were also reported by Ercan and Ayar Sensoy and showed range of embryo development varied from 0 to 7.69%, but their respective development into the normal regenerants was reportedly very low (12 plants)[25].

Effect of donor plant age[26], growing conditions[27], growing season[28], plant growth regulators[29] and genotype of the donor plant have reported to affect the efficiency and success of microspore embryogenesis; however, genotype is a main factor affecting the successful pepper microspore embryogenesis[30-33].

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Appropriate microspore development stage for *in vitro* cultivation and significant effect of stress treatment to switch the microspore gametophyte to sporophyte embryo stage has also been determined[34-35]. However, the influence of culture conditions on anther cultivation and culture medium composition can be optimized in order to increase the effectiveness of microspore embryogenesis[36-38]. The aim of this experiment was to study the effect of duration time of anther cultivation and composition of regeneration medium to increase the embryo production and number of regenerants.

Materials and Methods

Plant Material

The present research work was carried out in two consecutive years with two pepper varieties Stryama and Zlaten Medal 7. Donor plants were grown under glasshouse conditions from April to October.

Experimental Evaluation

Flower buds providing anthers with microspores from late uninucleate stage were surface sterilized in 5% calcium hypochlorite for 20 min and rinsed thrice with sterile dH_2O. Anthers were incubated on induction culture medium containing micro- and macro salts by Murashige and Skoog[39], vitamins by Gamborg *et al*[40], 0.1 mg L^{-1} 2, 4-dichlorophenoxy acetic acid, 0.1 mg L^{-1} kinetin (K), 0.005 mg L^{-1} biotin, 0.1 mg L^{-1} glycine, 0.04 mg L^{-1} vitamin B12, 0.5 mg L^{-1} ca-pantotenat, 30 g L^{-1} sucrose and 0.7% agar. Petri dishes with anthers were treated in darkness at 35 ± 1°C for the first eight days followed by incubation on the same medium without application of growth regulators after 12 days and named regeneration medium (12 medium) as a control.

The effect of incubation period on induction medium and different regeneration medium variants were analyzed for improvement of embryo induction and plantlets regeneration (number and conversion ratio). First experiment was combined with three concentrations of cytokinin kinetin in regeneration medium and five different duration times on incubation medium (12, 14, 16, 18 and 20 days). Culture medium variant 12-1 contained 0.1 mg L^{-1} K, 12-2 with 0.2 mg L^{-1} K and 12-3 with 0.3 mg L^{-1} K. Second experiment was combined by regeneration medium supplemented with 0.25 mg L^{-1} zeatin and 0.1 mg L^{-1} indole-3-acetic acid (IAA) (variant 12-4), and with 0.5 mg L^{-1} zeatin and 0.1 mg L^{-1} IAA (variant 12-5), and different time points during the incubation medium after 12, 14, 16, 18 and 20 days. Both experiments were carried out with 20 explants in 10 replications for each incubation period, regeneration medium variant and genotype.

The ploidy level of the obtained plant-regenerants was determined by flow cytometry (Partec PA-2, Germany) and chromosomes observation. Flow cytometry analysis was performed by chopped newly growing leaves with 1 mL of nuclei extraction buffer (Solution A), filtered through a 50 µm CellTrics^R filter (Partec, Germany) and mixed with DAPI staining buffer (Solution B).

Statistical Analysis

Experimental data was calculated by Duncan's multiple range test to confirm significant differences among the means. Three-way analysis of variance (ANOVA) was applied to show the effect of genotype, culture medium and duration period, and interaction between main factors on embryo formation frequency and plants regeneration was also tested.

Results and Discussion

Results from this study clearly demonstrated positive influence of prolonged anthers incubation period and induction medium on embryo formation and plantlets regeneration (Fig. 1). Highest frequency of embryogenesis was observed from variety Stryama at 16 days cultivation (21.17%) whereas after 20 days treatment (8.08%) for Zlaten Medal 7. Highest percentage of developed embryos into plants was recorded for 20 day induction period in both varieties. As a result of this study the highest embryo formation frequency was obtained in culture medium variant 12-1 and 12-4 (containing low K concentration and combination of zeatin and IAA), whereas highest regeneration frequency was seen in control medium for both varieties (Fig. 2).

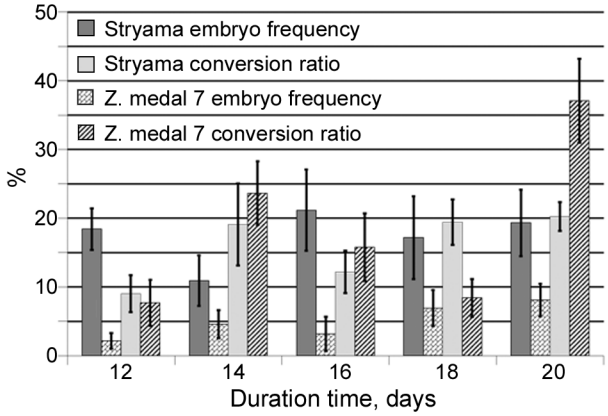

Fig. 1 — Influence of duration time on effectiveness of embryo formation and plantlets regeneration in pepper anther culture

Individual reaction of the genotypes in regard to incubation period and regeneration medium variants is presented in Table 1 & 2, respectively. Under the control conditions, embryo development was observed only in the anthers of Stryama. In this genotype low K concentration 0.1 mg L^{-1} stimulated embryo induction and plant regeneration for 14 and 18 day's incubation time. After 16 days treatment a better reaction was registered in culture medium supplemented with 0.3 mg L^{-1} K, while for 12 and 20 days treatments a better reaction was reported in control medium variant without plant growth regulators. Positive effect of 0.1 mg L^{-1} K in regeneration medium of Zlaten Medal 7 was registered at incubation time of 18 days, while regeneration medium without growth regulators stimulated the embryogenesis at 14, 16 and 20 days. On regeneration medium 12-2 higher embryogenesis was recorded in anther cultivated for 12 days. In conclusion, 14 day long incubation and control regeneration medium improved embryo conversion ratio in Stryama, but the highest embryo formation frequency and number of plant-regenerants were registered after 18 days incubation period in medium variant 12-1. Better reaction in Zlaten Medal 7 was achieved at prolonged incubation (20 days) and control regeneration medium (12).

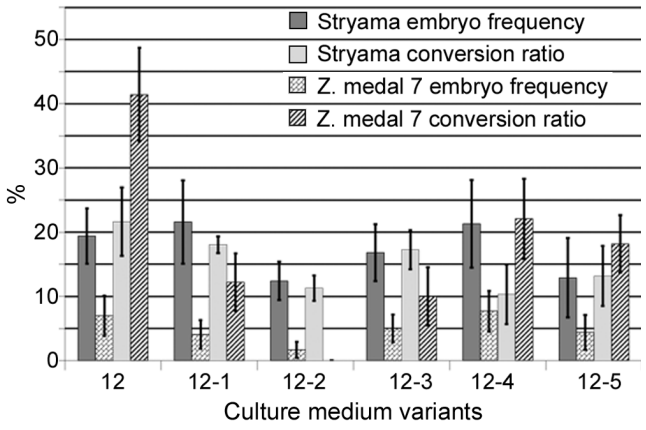

Fig. 2 — Effect culture medium variants on effectiveness of embryo formation and plantlets regeneration in pepper anther culture

After explants cultivation in regeneration medium supplemented with zeatin and IAA the results for Zlaten Medal 7 showed significant increase of androgenic frequency in medium variant 12-4, compared to variant 12-5 (Table 2). In anthers of Stryama same clear tendency has not been established. Higher embryo formation frequency in regeneration medium variant 12-5 was obtained at 12 and 20 days incubation period, while in variant 12-4 – after 14, 16 and 18 days. Regeneration frequency was higher on medium variant 12-4 at 18 and 20 days, while the explants subcultured in medium variant 12-5 reacted

Table 1 — Influence of incubation time and kinetin concentration on effectiveness of pepper anther culture

Duration time (days)	Medium	Reacted anthers		Formed embryos		Conversion ratio		Reacted anthers		Formed embryos		Conversion ratio	
		(No)	(%)	(No)	(%)	(No)	(%)	(No)	(%)	(No)	(%)	(No)	(%)
Genotype		Stryama						Zlaten Medal 7					
12	12	11	5.5	54	27.0 b-d	4	7.41 ef	0	0.0	0	0.0 g	0	0.00 c
	12-1	10	5.0	39	19.5 e-g	7	17.95 b-e	3	1.5	9	4.5 de	2	22.22 bc
	12-2	6	3.0	23	11.5 h-j	3	13.04 c-f	3	1.5	13	6.5 cd	0	0.00 c
	12-3	9	4.5	51	25.5 b-e	5	9.80d -f	1	0.5	4	2.0 fg	0	0.00 c
14	12	11	5.5	40	20.0 e-g	16	40.00 a	3	1.5	5	2.5e-g	0	0.00 c
	12-1	8	4.0	44	22.0 c-f	6	13.64 c-f	1	0.5	2	1.0 fg	0	0.00 c
	12-2	4	2.0	7	3.5 k	1	14.29 c-f	1	0.5	1	0.5 fg	0	0.00 c
	12-3	2	1.0	12	6.0 jk	2	16.67 b-f	1	0.5	3	1.5 fg	0	0.00 c
16	12	11	5.5	32	16.0 f-h	6	18.75 b-e	3	1.5	31	15.5 a	5	16.13 bc
	12-1	8	4.0	38	19.0 e-g	7	18.42 b-e	2	1.0	3	1.5 fg	0	0.00 c
	12-2	3	1.5	20	10.0 h-k	1	5.00 f	1	0.5	1	0.5 fg	0	0.00 c
	12-3	11	5.5	57	28.5 bc	12	21.05 b-d	1	0.5	3	1.5 fg	1	33.33 b
18	12	4	2.0	10	5.0 jk	2	20.00 b-e	2	1.0	7	3.5 ef	0	0.00 c
	12-1	18	9.0	88	44.0 a	18	20.45 b-d	4	2.0	25	12.5 b	3	12.00 bc
	12-2	11	5.5	43	21.5 d-g	4	9.30 d-f	0	0.0	0	0.0f g	0	0.00 c
	12-3	7	3.5	30	15.0 g-i	5	16.67 b-f	4	2.0	24	12.0 b	1	4.17 c
20	12	15	7.5	58	29.0 b	14	24.14 bc	5	2.5	27	13.5ab	24	88.89 a
	12-1	2	1.0	7	3.5 k	1	14.29 c-f	1	0.5	2	1.0 ef	0	0.00 c
	12-2	7	3.5	31	15.5 f-i	5	16.13 b-f	1	0.5	2	1.0 ef	0	0.00 c
	12-3	4	2.0	18	9.0 i-k	5	27.78 b	4	2.0	16	8.0 c	3	18.75 bc

a, b, c…. Values in columns followed by different letter are significantly different at $p < 0.05$, Duncan's Multiple Range Test

Table 2 — Influence of incubation time and zeatin concentration on effectiveness of pepper anther culture

Duration time (Days)	Medium	Reacted anthers		Formed embryos		Conversion ratio		Reacted anthers		Formed embryos		Conversion ratio	
		(No)	(%)	(No)	(%)	(No)	(%)	(No)	(%)	(No)	(%)	(No)	(%)
Genotype		Stryama						Zlaten Medal 7					
12	12-4	9	4.5	17	8.5^{ef}	0	0.00^{d}	0	0.0	0	0.0^{b}	0	0.00^{d}
	12-5	4	2.0	37	18.5^{d}	1	2.70^{d}	0	0.0	0	0.0^{b}	0	0.00^{d}
14	12-4	5	2.5	27	13.5^{de}	0	0.00^{d}	7	3.5	23	11.5^{a}	9	39.13^{a}
	12-5	1	0.5	1	0.5^{g}	0	0.00^{d}	9	4.5	21	10.5^{a}	4	19.05^{b}
16	12-4	17	8.5	93	46.5^{a}	3	3.23^{d}	0	0.0	0	0.0^{b}	0	0.00^{b}
	12-5	4	2.0	14	7.0^{f}	2	14.29^{c}	0	0.0	0	0.0^{b}	0	0.00^{b}
18	12-4	5	2.5	27	13.5^{de}	9	33.33^{a}	9	4.5	27	13.5^{a}	3	11.11^{c}
	12-5	6	3.0	8	4.0^{fg}	2	25.00^{b}	0	0.0	0	0.0^{b}	0	0.00^{b}
20	12-4	14	7.0	49	24.5^{c}	10	20.41^{b}	9	4.5	27	13.5^{a}	5	18.52^{b}
	12-5	26	13.0	69	34.5^{b}	12	17.39^{c}	5	2.5	23	11.5^{a}	4	17.39^{b}

a, b, c…. Values in columns followed by different letter are significantly different at $p < 0.05$, Duncan's multiple range test.

Table 3 — Three-way analysis of variance on embryo formation frequency and plantlets regeneration depending on genotype, regeneration medium and duration time

Source of variation	Relative effect size (% of total variance)	
	Formed embryos	Plant-regenerants
Genotype (A)	31.30***	2.41***
Culture medium (B)	5.61***	8.98***
Duration time (C)	3.35***	9.69***
A x B	1.55***	2.20*
A x C	3.61***	3.93***
B x C	27.62***	25.91***
A x B x C	22.78***	28.16***
Error	4.18	18.72

Level of significance: *p < 0.05, ** p < 0.01, *** p < 0.001

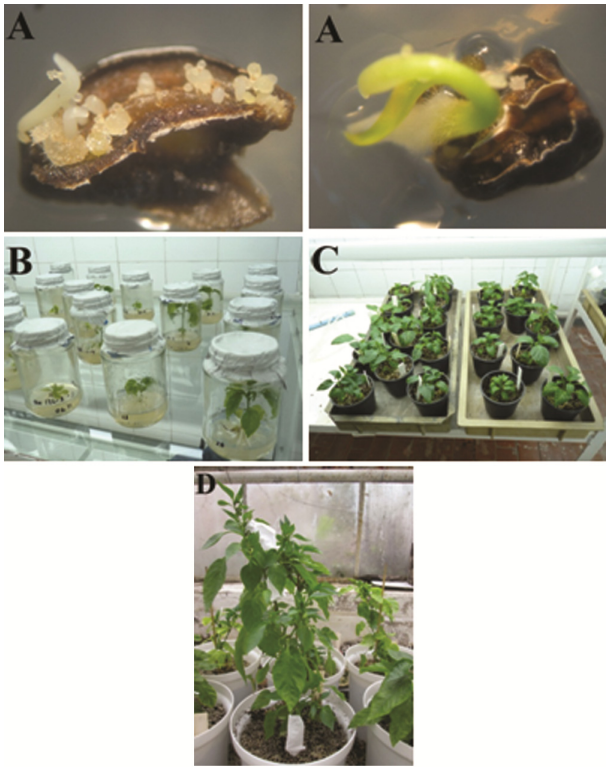

Fig. 3 (A-D) — Anther culture of pepper; (A) Direct embryogenesis; (B) Plant-regenerants; (C) Adapted plants & (D) Acclimatized plants

better in 12 and 16 days. A lack of regeneration was registered in the embryos of Stryama cultivated on medium 12-4 in 12 and 14 incubation time and on medium 12-5 for 14 days. However, it must be noted that in Stryama the number of embryos developed into normal plants in medium supplemented with zeatin and IAA was considerably lower in comparison to medium variants with K, but the embryo induction was with the same values. The combination of zeatin and IAA in regeneration medium stimulates callus formation and makes possible somatic origin of some of the obtained regenerants.

Data from three way ANOVA indicated that genotype effect on embryo formation in pepper anther culture was highly significant (31.30%), but the interactions of culture medium x incubation time and genotype x culture medium x incubation time also had an influence on the process (27.62% and 22.78%, respectively) (Table 3). In regard to plantlets regeneration, interaction between three studied factors and culture medium x incubation time (28.16% and 25.91% respectively) dominated over other factors (Table 3). These results suggest that after embryonic induction appropriate conditions for further embryo development into the plant-regenerants is needed to be found. Depending on the culture conditions, embryos converted into normal plants ranged from 0% to 88.89% (Fig. 3A, B). Successfully adapted and

subsequently acclimatized plants were 211 (92.9%) from 227 obtained plants (Fig. 3C, D). Flow cytometry analysis of ploidy level proved 40.8% haploid and 59.2% diploid regenerants.

In the past twenty years, considerable numbers of experiments with pepper anther culture have been conducted, but an efficient protocol for genotype independent haploid induction is still lacking. Specificity of the genotype androgenic answer ranged from 0 to 75 plants per 100 anthers[41]. Shrestha *et al* reported only 109 plants from 6100 cultivated anthers with embryogenic frequency of 1.78%[42]. Although a large number of embryos were obtained but the percentage of developed embryos into plantlets was low. This demands new approaches to increase double-haploids for practical application.

Moreover, the changes in the culture procedures can have an extreme effect on effectiveness of androgenesis. According to Supena and Custers[23], period till the globular stage is important for development of normal-looking or complete embryo. In contrast, Seguí-Simarro *et al* suggests that the investigation should mainly focused beyond globular stages when embryo elongates and matures so that the embryo quality can be improved. The results of this research work are in accordance with the data of Seguí-Simarro *et al* and proved that incubation period significantly increases the percentage of normal developed embryos into plants in comparison to the studied variants of regeneration medium.

Previous studies have shown that the increasing incubation time of anther cultivation from 12 to 14 days enhances the embryo formation efficiency[43]. According to Irikova *et al* the longer cultivation of explants on induction medium revealed the ability of direct embryogenesis on the tested genotypes[44], but further development of the embryos into plantlets is highly dependent on regeneration medium composition. Positive effect of higher kinetin concentration on regeneration medium was reported by Gemesne *et al*[45]. Parra-Vega *et al*[46] established that shorter duration in 35ºC (4 days) increased the embryo production compared to prolonged treatments (8 days). Furthermore, shorter incubation treatment in darkness decreased the callus formation whereas in regeneration process the relationship was reversed. Nowaczyk and Kisiała have established that 12 days incubation and 0.2 mg L^{-1} K on R1 medium increased the androgenic embryos development, while prolonged treatment to 14 days corresponded to more embryos when R1 medium were supplemented with 0.1 mg L^{-1} K. Olszewska *et al* observed the highest effectiveness of androgenesis after 16 days in CP medium and R1 medium with 0.1 mg L^{-1} K and 0.3 mg L^{-1} K at 12 and 14-day-long anther incubation, but the reaction depended on genotype. Consequently, the possibility for applying different culture conditions allows the choice of the most convenient for each variety.

In conclusion, development of effective and applicable protocol for pepper anther culture is important to determine duration time of anther cultivation on induction medium and regeneration medium, particularly the concentration and combination of plant growth regulators. Prolonged treatments of anthers on incubation medium significantly increased the microspore embryogenesis in studied pepper varieties, but corresponded with low K concentration in regeneration medium. Therefore, combination of induction time and regeneration media is important for embryo formation and subsequent development of plant regenerants.

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