

**IN VITRO ANSWER OF BULGARIAN PEPPER
(CAPSICUM ANNUUM L.) VARIETIES**

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Callusogenesis and regeneration ability of cotyledon and hypocotyl explants from three Bulgarian pepper varieties in MS basal medium supplemented with 1-3mg/l BAP, 1.0mg/l IAA and 0.5mg/l GA3 was studied. In the different variants of culture medium was registered high level of callusogenesis and organogenesis in both type of explants from the all varieties. The highest percentage of plant-regenerants is established in cotyledon explants (from 3.3 to 18.3) in variant 3 of the culture medium containing 3mg/l BA. In the process of micropropagation by stem explants of the same studied pepper varieties the addition of the vitamins C, B₁₂, Casein hydrolysate and Ferulic acid had a stimulation effect on the plant growth in height and rooting. In result of anther cultivation from three pepper varieties and four breeding lines the highest percentage of embryo structure formation was registered in varieties Albena and Strjama (12.0 and 13.8 respectively). The Bulgarian peppers are recalcitrant and their *in vitro* answer is different depending from the explant type, genotype and the culture media composition.

Key words: pepper, callusogenesis, organogenesis, embryogenesis, plant-regeneration, micropropagation, explant type, culture media, genotype

INTRODUCTION

Pepper (*Capsicum annuum* L.) is one of the most important vegetable crops cultivated in the Balkan region and also all over the world. In Bulgaria there are an extraordinary genetic diversity of local forms and varieties typical for the region with specific and unique characteristics as color, shape and taste. They are an excellent base for breeding of well adapted F₁ hybrids. The conventional breeding and propagation of pepper cultivars and hybrids is a time consuming process with problems in open pollination, genetic impurity, sterility and often low yield of seeds. The development of effective pepper regeneration *in vitro* system is aimed to: 1) micropropagation of valuable for the breeders genetic material as male sterile forms, mutants, F₁ hybrids etc. (ZHENJIU S. and WANG M., 1990; CHEN *et al.*, 2005); 2) production of transgenic plants (STEINITZ B., 1999; LI *et al.*, 2003); 3) generation of haploids and doubled haploids (GYULAI *et al.*, 2000; IRIKOVA T. and RODEVA V., 2004).

It is well known that the current process of mass regeneration of pepper *in vitro* is far from complete understanding and rather underdeveloped (SHAO W. and CAPONETTI, 1993; RODEVA V. and GROZEVA S., 2003) what is an obstacle for using of the modern biotechnological methods in breeding of this vegetable crop.

The purpose of our work is to study the *in vitro* callusogenesis, regeneration ability and plantlet formation from different organ explants of Bulgarian pepper varieties.

MATERIAL AND METHODS

In the period of years 2004-05 seeds from three Bulgarian pepper varieties - Strjama, Hebar and Kurtovska kapija 1619, were surface sterilized and germinated on MS basal medium (MURASHIGE and SKOOG, 1962) in the condition of growth chamber - 25°C ±1°C, 14 hours photoperiod and 4000 lux provided by white fluorescent tubes. Cotyledon and hypocotyl explants from 8-10-old seedlings were cultivated in 9-cm Petri dishes on the medium supplemented with major and minor salts by MS, vitamins as in B5 (GAMBORG *et al.*, 1968), 1-3mg/l 6-Benzyladenine (BA), 1.0mg/l Indolyl-3-acetic acid (IAA), 0.5mg/l Gibberellic acid (GA3), 2% Sucrose and 0.7% Agar. The pH of the medium was adjusted to 5.8 before autoclaving. For the different variants 20 explants in 3 replications were cultivated of each of the genotype. The callusogenesis and regeneration capacity (% explants with regeneration) was reading at the end of 90-days cultivation period. For the micropropagation purposes *in vitro* plants were cut to shoot tips and stem parts with axillary buds. The explants were cultivated on 3 variants of MS basal medium different in concentration of Vitamins C, B₁₂, Casein hydrolysate and Ferulic acid. The coefficient of micropropagation is calculated on the basis of obtained microplants from 20 explants for the period of 30 days. Anthers with pollen in uninucleate microspore stage from three pepper varieties (Albena, Strjama, Gorogled) and four breeding lines (N1957, N53, N73 and N94) were

selected from sterilized buds and cultivated at +35°C for 8 days on optimized induction medium with macro- and microsalts by MS, vitamins by B5, vitamin B12 - 0.04mg/l. 2.4 - D 0.004mg/l and kinetin - 0.1mg/l. Later the anthers were incubated on the same medium without growth regulators in the condition of growth chamber. After 5-6 weeks of incubation the numbers of developed embryos were recorded. The results from the regeneration experiment were calculated by two-factor analysis of variance GENCHEV *et al.* (1975).

RESULTS AND DISCUSSION

The data from Table I. 1 show that the two types of cultivated explants from the three studied varieties develop callus, adventitious buds and organogenic structures at the wounds of explants regardless of the medium composition. Although all explants produced callus this process run with different intensity depending of the variety and the culture medium and take 20-40 days from the beginning of the cultivation period.

Table 1. - In vitro answer of cotyledon and hypocotyl explants from three pepper varieties

Culture medium	Hypocotyls Reacted explants %			Cotyledons Reacted explants %		
	Calluso- genesis	Organo- genesis	Regene- ration	Calluso- genesis	Organo- genesis	Regene- ration
Variety Strjama						
1	100	60.0	1.6	100	50.0	3.3
2	100	50.0	3.3	100	70.0	3.3
3	100	66.7	5.0	100	70.0	6.7
average	100	58.9	3.3	100	63.3	4.4
Variety Hebar						
1	100	53.3	0.0	100	83.3	1.6
2	100	70.0	0.0	100	80.0	5.0
3	100	91.6	3.3	100	100.0	18.3
average	100	71.7	1.1	100	87.8	8.3
Variety Kurtovska kapija 1619						
1	100	43.3	0.0	100	60.0	0.0
2	100	50.0	0.0	100	65.0	1.6
3	100	50.0	0.0	100	65.0	5.0
average	100	47.8	0.0	100	63.3	2.2
Average for the 3 varieties	100	59.4	1.5	100	71.5	5.0

Note: 1 - 1.0 mg/l BA + 1.0 mg/l IAA + 0.5 mg/l GA3,

2 - 2.0 mg/l BA + 1.0 mg/l IAA + 0.5 mg/l GA3,

3 - 3.0 mg/l BA + 1.0 mg/l IAA + 0.5 mg/l GA3

The explants of varieties Strjama and Hebar responded better and earlier than these of variety Kurtovska kapija. This dependency was observed also in the process of organogenesis. The better answer in the two types of explants was

obtained in variety HEBAR (71.7 and 87.8% respectively) and variety Strjama (58.9 and 63.3% respectively) compared with variety Kurtovska kapija (47.8 and 63.3% respectively). Although most of the explants react with morphogenesis (from 43.3 to 100%) only small part of the formed adventitious buds in the three studied varieties developed to elongated plant-regenerants (from 0.0 to 18.3%). The most of the buds formed later pale, leaflike structures and leaves as it is observed from other authors also (SZASZ *et al.*, 1995).

The data from the Tabl.1 prove that the differences in the organogenic and regeneration ability between the two types of explants are not significant, but the reaction of the cotyledons is visible better (71.5% compared to 59.4% respectively). The frequency of the obtained plant-regenerants is highest in variant 3 of the studied culture medium what show that the increasing of BA concentration to 3.0mg/l in our experiment promote shoot induction (Fig.1.). The regeneration capacity of the studied pepper varieties is comparatively low for efficient regeneration system what is in accordance with the results published of the most of the authors and probably is due to the specific nature of these genotypes and need the optimization of the culture conditions (CHRISTOPHER and RAJAM, 1996; CHEN *et al.*, 2005). This is proved also from the statistical analysis - the culture media and genotypes are the base factors influencing on the regeneration capacity of the studied varieties (Table 2.).

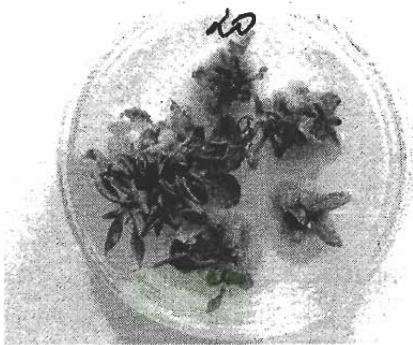


Fig. 1. - Organogenesis and regeneration in cotyledon explants from variety Hebar

Table 2. - Analysis of the influence of culture media and genotype on regeneration capacity in cotyledon explants from pepper varieties Strjama, Hebar and Kurtovska kapija

Source of variation	df	SS	MS	F	Fcrit.
Culture medium - CM	2	301.8519	150.9259	16.3***	3.55
Genotype - G	2	201.8519	100.9259	10.9***	3.55
CM x G	4	203.7037	50.9259	5.5**	2.93
Error	18	166.6670	9.2592		
Total	26	874.0714			

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

The response of stem explants of the studied pepper varieties to the different combinations of vitamins and supplements in the culture medium is visible from the data of Table 3. The highest coefficient of micropropagation (from 1.90 to 2.15) in all varieties is registered in variant N20 containing Vitamins C and B₁₂. Casein hydrolysate and Ferulic acid which apparently promote plant growth in height, stimulate developing of lateral buds and rooting (Fig. 2.). There were not established significant differences in the coefficient of propagation among the studied genotypes when explants are cultivated in this variant. These results encourage the successful micropropagation of pepper *in vitro* by culture medium optimization.

Table 3. - *In vitro* micropropagation in three pepper varieties (*Capsicum annum L.*)

Culture medium	No of cultured explants	Plants obtained after 30 days of cultivation	
		No	Coefficient of propagation
Variety Strjama			
MS	20	22	1.10
17	20	23	1.15
19	20	35	1.75
20	20	40	2.00
Variety Hebar			
MS	20	26	1.30
17	20	33	1.65
19	20	36	1.80
20	20	38	1.90
Variety Kurtovska kapija 1619			
MS	20	20	1.00
17	20	23	1.15
19	20	26	1.30
20	20	43	2.15

Note: 17 - MS + 25 mg/l Vit.C + 5 mg/l Cas. Hydrolysate

19 - MS + 10 mg/l Vit.C + 100 mg/l Cas. Hydrolysate

20 - MS + 10 mg/l Vit.C + 0.4 mg/l Vit. B₁₂ + 100 mg/l Cas. Hydrolysate
+ 0.4 mg/l Ferulic acid



Fig. 2. - Micropropagation *in vitro* of variety Strjama in different medium variants

The data from Table 4. prove that the anthers from all studied pepper genotypes reacted with embryo structure formation with exception of line N 53 in

year 2004. The highest percentage of embryo structures is registered in varieties Strjama (13.8 and 7.0 respectively) and Albena (12.0 and 4.0 respectively) in the two experimental years (Fig. 3.). In the breeding lines the highest values are established in N 94 (6.0%) and N 1957 (3.4%). Although the highest percentage structures are formed in the studied varieties most of them didn't develop into plantlets. The percentage of the obtained plant-regenerants from the varieties is comparatively lower to these developed from the embryos of the studied lines what is proved from the correlation between the figures embryoid structures:obtained regenerants. These results can be explained with the specific *in vitro* answer of the different genotypes and the higher plasticity of the hybrid lines obtained with participation of the male sterile component.

Table 4. - Embryogenic answer in anther culture of different pepper genotypes

Genotype	Number of cultured anthers	Anthers with embryos %	Embryoid structure %	Obtaining regenerants %
2004				
1957	280	2,5	3,4	0,4
53	300	0,0	0,0	0,0
73	110	0,5	0,9	0,4
94	280	0,5	0,6	0,5
Average	970	0,84	1,22	0,32
Embryoid structures : obtained regenerants			1:3,8	
Gorogled	260	1,90	2,2	0,0
Albena	365	11,0	12,0	0,0
Strjama	230	10,9	13,8	2,2
Average	855	7,91	9,32	0,72
Embryoid structures : obtained regenerants			1:12,9	
2005				
1957	300	1,3	1,3	1,0
53	250	0,8	3,6	2,0
73	220	0,4	0,9	0,4
94	265	1,5	6,0	2,3
Average	1035	1,20	2,96	1,43
Embryoid structures : obtained regenerants			1:2,1	
Gorogled	200	5,0	5,0	0,7
Albena	350	2,0	4,0	0,6
Strjama	200	5,0	7,0	1,6
Average	750	4,00	5,33	0,98
Embryoid structures : obtained regenerants			1:5,4	



Fig. 3. Embryogenesis in anther culture of pepper (*Capsicum annuum* L.)

CONCLUSIONS

In the result of this study it was established that the hypocotyl and cotyledon explants from all varieties (Strjama, Hebar and Kurtovska kapija) studied for regeneration capacity reacted by callusogenesis and organogenesis. A highly recalcitrant nature of Bulgarian pepper varieties and variation by regeneration ability was registered. The cotyledon explants was superior compared to hypocotyls. The highest percentage of plant-regenerants (18,3) was obtained in variety Hebar in medium variant N3. In the process of micropropagation the addition of Vitamins C, B₁₂, Casein hydrolysate and Ferulic acid to MS basal medium stimulates the growth of stem explants in height and rooting. There were not established significant differences in the coefficient of propagation among the studied genotypes in the medium variant N20. In result of anther cultivation from three pepper varieties (Gorogled, Albena and Strjama) and four lines (N1957, N53, N73 and N94) the highest percentage of embryo structures was registered in varieties Strjama (13,8) and Albena (12,0). The percentage of the obtained plant-regenerants in result of androgenic embryogenesis in the studied varieties is comparatively lower to this developed from the embryos of the studied lines what is proved from the correlation between the figures embryoid structures:obtained regenerants. The established variations in the genotype response to *in vitro* cultivation emphasizing the need to standardize the protocols in each of them with commercial or breeding importance.

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IN VITRO ODGOVOR BUGARSKIH SORTI PAPRIKE (*CAPSICUM ANNUUM* L.)

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I z v o d

Kalusogeneza i sposobnost regeneracije eksplanta kotiledona i hipokotila tri sorte bugarske paprike na MS osnovnom medijumu kome je dodato 1-3mg/l BAP, 1,0mg/l IAA i 0,5mg/l GA3 je ispitivana. Na različitim varijantama medijuma utvrđen je visok nivo kalusogeneze i organogeneze u oba tipa eksplanta svi sorata. Najviši procenat regenerisanih biljaka je dobijeno u eksplantima kotiledona (od 3,3 do 18,3) u sorti 3 na medijumu koji sadrži 3mg/l BA. U procesu mikropropagacije stem eksplanta ispitivanih sorti dodatak vitamina C, B₁₂, Casein hidrolizate i Ferulic kiseline je imalo stimulativan efekat na visinu i formiranje korena. Kultivacijom antera tri sorte paprika i četiri linije najviši procenat formiranja embriona je registrovan u sortiama Albena i Strjama (12,0 i 13,8). Bugarska paprike su recalcitrant i njihov *in vitro* odgovor je različit u zavisnosti od tipa eksplanta, genotipa i sastava medijuma.

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