UDC 575.630
DOI: 10.2298/GENSR1402485G
Original scientific paper

IN VITRO PLANT REGENERATION OF TWO CUCUMBER (*Cucumis sativum* L.) GENOTYPES: EFFECTS OF EXPLANT TYPES AND CULTURE MEDIUM

Stanislava GROZEVA and Nikolay VELKOV

Maritsa Vegetable Crops Research Institute – Plovdiv, Plovdiv, Bulgaria

Grozeva S. and, N. Velkov (2014): *In vitro plant regeneration of two cucumber (Cucumis sativum L.) genotypes: effects of explant types and culture medium-*. Genetika vol 46, No2, 485-493.

The effect of different phytohormone concentrations on callusogenesis and organogenesis in two cucumber genotypes were studied. It was established that the rate of plant regeneration depends on genotype, explant type and culture medium. Hypocotyls were found to be more responsive than cotyledons in morphogenesis. *In vitro* planlet-regenerants have been obtained in hypocotyls explants on culture medium with 1.0 and 2.0 mgL^{-1} BA for cultivar Gergana and in 1.0 and 3.0 mgL^{-1} K – line 15B. Induction of regeneration in cotyledons were established only in cultivar Gergana on culture medium supplemented with 3.0 mgL^{-1} BA and in combination of 0.5 mgL^{-1}IAA.

Key words: cucumber**,** callusogenesis, genotype**,** plant growth regulators, organogenesis

INTRODUCTION

Conventional genetic improvement of cucumber is limited because it is time-consuming and leading to homogeneity and results in narrowing the genetic potential. *In vitro* methods especially plants tissue culture are an essential tool for creation of new genetic variation and unique recombination (PLADER *et al*., 1998; RODEVA *et al*., 2006; TODOROVA *et al*., 2013).

Development of rapid and efficient regeneration system can accelerate the process via somaclonal variation, *in vitro* mutagenesis and genetic engineering (NANASATO *et al*., 2013). Successful induction of plant regeneration in cucumber has been achieved from different explant types, genotypes and culture media (MOHINDDIN *et al*., 2005; SONG *et al*., 2007; VASUDEVAN *et al*., 2008; UGANDHAR *et al*., 2013). Plant growth regulator BA alone or in combination with low concentration of auxins IAA and NAA stimulate organogenesis and shoot induction (SELVARAJ *et al*., 2007; UGANDHAR *et al*., 2011). MOHINDDIN *et al*. (2005) and AYYAPPAN *et al*. (2006) proved that silver nitrate ($AgNO_3$) significant increase the frequency of shoot induction in different cucumber varieties. Better regeneration answer has been observed by USMAN *et al*.

***Corresponding author**:* Stanislava Grozeva, Department of Breeding, Variety Maintenance and Introduction, Maritsa Vegetable Crops Research Institute, Brezovsko shosse 32, 4003 Plovdiv, Bulgaria, Tel.: +35932952296, Fax: +35932960177, e-mail: stanislava_grozeva@abv.bg;

(2011) in leaf explants compared to cotyledons. In contrast, CURUK *et al*. (2003) and KOSE & KOÇ (2003) recommended cotyledons and hypocotyls as explant types. However cucumber regeneration via callus formation is still limited due to the low regeneration frequency and genotype dependence of the process (SELVARAJ *et al*., 2006).

This experimental work is aimed to study *in vitro* regeneration potential of the cotyledon and hypocotyl explants of two cucumber genotypes.

MATERIALS AND METHODS

The experiment was carried out in the "Maritsa" Vegetable Crops Research Institute – Plovdiv during 2012-2013. Two cucumber genotypes inbred line 15B and cultivar Gergana were used.

Seeds of the two cucumber genotypes were surface sterilized in 5% calcium hypochlorite solution for 1 hour and rinsing three-times and sterile dH_2O. After that the seeds were sown on MURASHIGE & SKOOG (1962) (MS). Explants of cotyledons (0.5 cm) and hypocotyls (1.0 cm) were excised from 5-7 days old *in vitro* grown seedlings. The explants were cultivated in Petri dishes on basal medium contained major and minor salts by MS, vitamins as in B5 medium (GAMBORG *et al*., 1968), 3% sucrose, 0.7% agar and supplemented with plant growth regulators N^6-Benzyladenine (1, 2 and 3 mgL^{-1}) (BA) and Kinetin (1, 2 and 3 mgL^{-1}) (K) alone or in combination with 0.5 mgL^{-1} Indolil-3-acetic acid (IAA).

The explants were incubated in growth chamber at 25ºC ± 1ºC temperature, a photosynthetic proton flux density (PPFD) of 200 μmol m^{-2} s^{-1}, 16/8 h photoperiod and subcultured at intervals of 15 days on the same medium. The experiment was carried out in three replications with 20 explants in each for the different genotypes, medium variant and explant type. The callusogenesis, organogenesis, regeneration frequency (% explants with regeneration) and number of regenerants per explant were examined for a period of 90 days.

The experimental data were calculated by Duncan's multiple range test to confirm statistical significant of difference among the means. Three-way analysis of variance were applied to show effect of genotype, culture medium and explant type, and interaction between main factors on callusogenesis and regeneration frequency. Hierarchical cluster analysis was applied to estimate similarities between studied variants.

RESULTS AND DISCUSSION

Data presented in Table 1 show that all cultured cotyledon explants from two studied genotypes reacted with callusogenesis on culture medium supplemented with BA (1-3 mgL^{-1}) and 0.5 mgL^{-1}IAA. Callus formation were not established in cotyledons on medium containing K or with combination of IAA in line 15B and in medium variant containing 3.0 mgL^{-1} K alone or in combination of 0.5 mgL^{-1} IAA in cultivar Gergana. In contrast, hypocotyls reacted with callusogenesis in all medium variants in ranged from 80.0% to 100% of cultivated explants. These results are supported by the data for three-way analysis that the process of callusogenesis influence mainly of explants types (33.17%) followed by culture medium and interaction of both factors (22.87% and 23.88% respectively) (Table 2).

Hypocotyls were found to be more responsive than cotyledons in organogenesis. In line 15B organogenic reaction in hypocotyls was registered from 0.0% to 100% of explants while in cultivar Gergana from 0.0% to 93.3%. In cotyledon explants of both genotypes organogenesis with the highest frequency was observed in culture medium supplemented with 3.0 mgL^{-1}BA

(41.7% and 76.7%). Organogenic reaction in cotyledons of line 15B was registered also in medium variant containing 2.0 mgL^{-1}BA and 3.0 mgL^{-1}BA + 0.5 mgL^{-1}IAA (33.3% and 10.0% respectively) while in cultivar Gergana in 1.0 mgL^{-1}BA and combination BA 1.0 and 3.0 mgL^{-1} with 0.5 mgL^{-1}IAA (15.0%, 25.0% and 66.7% respectively). According to MOHIUDDIN *et al*. (2005) regeneration capability of cucumber strongly depends on explants type and genotype even the culture conditions were optimal and established that distal cotyledons as less responsive. In contrast, different reaction between cotyledon and hypocotyls segments was not observed (UGANDHAR *et al*., 2011).

Table 1. Effect of culture medium on callusogenesis and organogenesis in cotyledon and hypocotyl explants by two cucumber genotypes

Explant types	Culture medium variants	15B		Gergana		15B		Gergana	
		Cotyledon				Hypocotyl			
Callusogenesis	1.0 mgL^{-1}BA	71.7	c	90.0	ab	100	a	100	a
	2.0 mgL^{-1}BA	80.0	bc	53.3	c	100	a	80.0	b
	3.0 mgL^{-1}BA	75.0	c	76.7	b	100	a	80.0	b
	1.0 mgL^{-1}K	0.0	d	86.7	ab	90.0	b	93.3	ab
	2.0 mgL^{-1}K	0.0	d	81.7	b	100	a	90.0	ab
	3.0 mgL^{-1}K	0.0	d	0.0	d	100	a	100	a
	1.0 mgL^{-1}BA + 0.5 mgL^{-1}IAA	100	a	100	a	100	a	100	a
	2.0 mgL^{-1}BA + 0.5 mgL^{-1}IAA	100	a	100	a	100	a	100	a
	3.0 mgL^{-1}BA + 0.5 mgL^{-1}IAA	93.3	ab	100	a	100	a	100	a
	1.0 mgL^{-1}K + 0.5 mgL^{-1}IAA	0.0	d	50.0	c	100	a	100	a
	2.0 mgL^{-1}K + 0.5 mgL^{-1}IAA	0.0	d	56.7	c	100	a	100	a
	3.0 mgL^{-1}K + 0.5 mgL^{-1}IAA	0.0	d	0.0	d	100	a	100	a
Organogenesis	1.0 mgL^{-1}BA	0.0	d	15.0	d	23.3	d	93.3	a
	2.0 mgL^{-1}BA	33.3	b	0.0	e	10.0	ef	80.0	b
	3.0 mgL^{-1}BA	41.7	a	76.7	a	10.0	ef	0.0	e
	1.0 mgL^{-1}K	0.0	d	0.0	e	90.0	b	0.0	e
	2.0 mgL^{-1}K	0.0	d	0.0	e	50.0	c	0.0	e
	3.0 mgL^{-1}K	0.0	d	0.0	e	100	a	0.0	e
	1.0 mgL^{-1}BA + 0.5 mgL^{-1}IAA	0.0	d	25.0	c	6.7	ef	83.3	b
	2.0 mgL^{-1}BA + 0.5 mgL^{-1}IAA	0.0	d	0.0	e	13.3	e	36.7	d
	3.0 mgL^{-1}BA + 0.5 mgL^{-1}IAA	10.0	c	66.7	b	0.0	f	53.3	c
	1.0 mgL^{-1}K + 0.5 mgL^{-1}IAA	0.0	d	0.0	e	50.0	c	0.0	e
	2.0 mgL^{-1}K + 0.5 mgL^{-1}IAA	0.0	d	0.0	e	50.0	c	3.3	e
	3.0 mgL^{-1}K + 0.5 mgL^{-1}IAA	0.0	d	0.0	e	50.0	c	3.3	e

a,b,c….$p \leq 0.05$ Duncan's Multiple Range Test

Table 2. Three-way analysis of variance and influence of variation factors on the callusugenes depend on genotypes, explant types and culture media

Source	Sum of Squares	df	Mean Square	F	Sig.	Power of influence (η%)
Genotype (A)	188995.7	1	4021.2	60.48	***	1.67
Explant type (B)	831896.0	1	831896.0	12511.02	***	33.17
Culture medium (C)	3258.5	11	3258.5	49.01	***	22.87
A x B	64812.7	1	64812.7	974.73	***	3.31
A x C	44685.2	11	4062.3	61.09	***	6.72
B x C	6466.8	11	6466.8	97.26	***	23.88
A x B x C	13122.7	11	1193.0	17.94	***	5.12
Error	46651.9	96	4241.1			3.27
Total	195379.0	143				

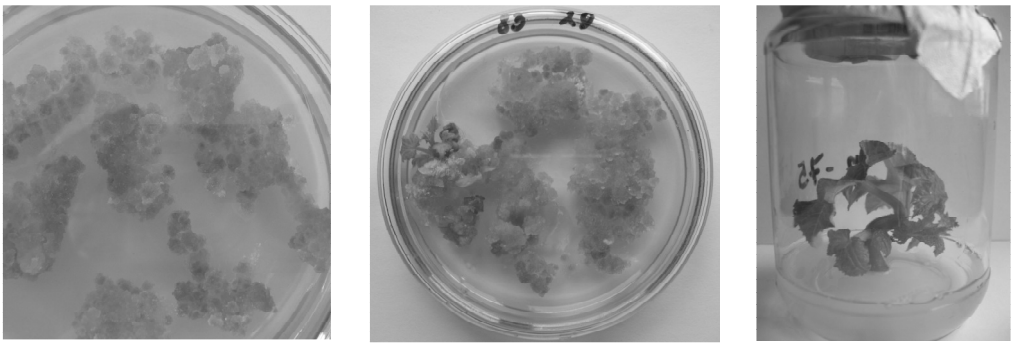
R Squared = 0.967 (Adjusted R Squared = 0.951)

Table 3. Regeneration efficiency in two cucumber genotypes

Explant types	Culture medium variants	15B	Gergana		15B		Gergana	
		Cotyledon			Hypocotyl			
Regeneration	1.0 mgL^{-1}BA	0.0	15.0	c	0.0	b	73.3	a
	2.0 mgL^{-1}BA	0.0	0.0	d	0.0	b	10.0	b
	3.0 mgL^{-1}BA	0.0	33.3	b	0.0	b	0.0	b
	1.0 mgL^{-1}K	0.0	0.0	d	13.3	a	0.0	b
	2.0 mgL^{-1}K	0.0	0.0	d	0.0	b	0.0	b
	3.0 mgL^{-1}K	0.0	0.0	d	10.0	ab	0.0	b
	1.0 mgL^{-1}BA + 0.5 mgL^{-1}IAA	0.0	6.7	cd	0.0	b	0.0	b
	2.0 mgL^{-1}BA + 0.5 mgL^{-1}IAA	0.0	0.0	d	0.0	b	3.3	b
	3.0 mgL^{-1}BA + 0.5 mgL^{-1}IAA	0.0	61.7	a	0.0	b	0.0	b
	1.0 mgL^{-1}K + 0.5 mgL^{-1}IAA	0.0	0.0	d	0.0	b	0.0	b
	2.0 mgL^{-1}K + 0.5 mgL^{-1}IAA	0.0	0.0	d	0.0	b	0.0	b
	3.0 mgL^{-1}K + 0.5 mgL^{-1}IAA	0.0	0.0	d	0.0	b	0.0	b
Regenerants/ Explant	1.0 mgL^{-1}BA	0.00	0.15	c	0.00	b	0.90	a
	2.0 mgL^{-1}BA	0.00	0.00	c	0.00	b	0.10	b
	3.0 mgL^{-1}BA	0.00	0.33	b	0.00	b	0.00	b
	1.0 mgL^{-1}K	0.00	0.00	c	0.13	a	0.00	b
	2.0 mgL^{-1}K	0.00	0.00	c	0.00	b	0.00	b
	3.0 mgL^{-1}K	0.00	0.00	c	0.10	ab	0.00	b
	1.0 mgL^{-1}BA + 0.5 mgL^{-1}IAA	0.00	0.07	c	0.00	b	0.00	b
	2.0 mgL^{-1}BA + 0.5 mgL^{-1}IAA	0.00	0.00	c	0.00	b	0.03	b
	3.0 mgL^{-1}BA + 0.5 mgL^{-1}IAA	0.00	0.75	a	0.00	b	0.00	b
	1.0 mgL^{-1}K + 0.5 mgL^{-1}IAA	0.00	0.00	c	0.00	b	0.00	b
	2.0 mgL^{-1}K + 0.5 mgL^{-1}IAA	0.00	0.00	c	0.00	b	0.00	b
	3.0 mgL^{-1}K + 0.5 mgL^{-1}IAA	0.00	0.00	c	0.00	b	0.00	b

a,b,c….$p \leq 0.05$ Duncan's Multiple Range Test

Statistical differences in regeneration potential were established in the explants depending on the cucumber genotypes and medium variant (Table 3). Regeneration ability varied from 0.0% to 73.3%. The highest regeneration activity was observed in hypocotyls from cultivar Gergana of medium containing 1.0 mgL^{-1}BA (73.3%) and in cotyledons of medium variant containing 3.0 mgL^{-1}BA + 0.5 mgL^{-1}IAA (61.7%) (Fig.1). Regeneration answer was not established in cotyledon explants of line 15B in all medium variants. Data from three-way analysis of variance indicated that regeneration ability strongly influence by interaction of both factors genotype and culture medium variant (24.16%), while effect of explants type was not statistically proven (Table 4).



callusogenesis regeneration Plant-regenerant

Figure 1. In vitro plant regeneration in hypocotyls of cultivar Gergana

Table 4. Three-way analysis of variance and influence of variation factors on the regeneration depend on genotypes, explant types and culture media

Source	Sum of Squares	df	Mean Square	F	Sig.	Power of influence (η%)
Genotype (A)	29638.9	1	630.6	21.62	***	6.24
Explant type (B)	3211.1	1	3211.1	110.10	ns	0.01
Culture medium (C)	2025.0	11	2025.0	69.43	***	20.50
A x B	2.8	1	2.8	0.10	*	0.55
A x C	6651.4	11	604.7	20.73	***	24.16
B x C	177.8	11	177.8	6.10	***	20.22
A x B x C	7837.5	11	712.5	24.43	***	19.68
Error	6559.7	96	596.3			8.63
Total	32438.9	143				

R Squared = 0,914 (Adjusted R Squared = 0,871)

The highest number of plantlets/explant in cultivar Gergana was obtained in hypocotyl segments on culture medium with 1.0 mgL^{-1}BA and in cotyledons on medium variant with 3.0 mgL^{-1}BA + 0.5 mgL^{-1}IAA (0.90 and 0.75 respectively). In line 15B developed plantlets was established only in hypocotyls segments on medium variants containing 1 and 3 mgL^{-1}K (0.13 and 0.10 respectively) (Table 3).

UGANDHAR *et al*. (2011) reported that maximum number of plantlet regeneration was induced in culture medium supplemented with 3.0 mgL^{-1} BA and 0.5 mgL^{-1} IAA. The authors suggested that low level of IAA might have triggered the action of BA/K in a proper way for inducing more number of shoots per explants. The results obtained in the present work established different reaction of both explant types and combination 3.0 mgL^{-1} BA and 0.5 mgL^{-1} IAA was better for cotyledon while 1.0 mgL^{-1} BA or K – for hypocotyls. Probably the genotype is a main factor influence the regeneration process. Differences observed in regeneration frequency in the both explant types may be due the influence of endogenous growth regulators, the position of explant in the plant, and the tissue they consist. ANANTHAKRISHNAN *et al*. (2003) established small shoots and buds regenerated only on the most proximal cotyledon edge in *Cucurbita pepo*. Position-dependent of regeneration capacity of explants was proved in *Cucumis sativum* (MOHIUDDIN *et al*., 2005).

On the base of established differences in response of the two explant types and for define similarities/dissimilarities in reaction of the two cucumber genotypes (Gergana and 15B) in relation of callusogenesis, organogenesis and regeneration processes, of 12 culture medium variants it was performed cluster analysis. The studied culture medium variants and cucumber genotypes were distributed in two groups according to their distances (Fig. 2). The first group consisted of two types of culture medium 3 mgL^{-1} BA and 3 mgL^{-1} BA +0.5 IAA on line 15B. The second group included all other medium variants. The results show that the medium variants contained in the first cluster are more perspective for optimization of regeneration process in cotyledon explants in different cucumber genotypes.

The response of the hypocotyl segments was different (Fig. 3). It was observed two clusters, but the first contained only one variant (1 mgL^{-1} BA, cultivar Gergana) where regeneration processes were with the highest frequency. The second cluster was divided into two subgroups. The first subgroup included variants 1 and 3 mgL^{-1} K in 15B and 2 mgL^{-1} BA in Gergana. All other culture medium variants formed the second subgroup. The first subgroup was characterized by good callusogenesis and poor ability for organogenesis and regeneration, while the second subgroup comprised those medium variants in which were not induced regeneration reactions or the answer was sporadic. The results indicate that the experimental work should be continue for development of additional combinations associated with variants contained in the first cluster.

As a result of this study different regeneration response of hypocotyl and cotyledon explants compare to culture medium variants were established. Cultivar Gergana displayed the highest regeneration response in cotyledon explants on culture medium supplemented with 3.0 mgL^{-1} BA and 0.5 mgL^{-1} IAA, while in hypocotyls on medium variant containing 1.0 mgL^{-1} BA. In line 15B regeneration of plantlets were obtained only in hypocotyls segments on medium variants with 1.0 and 3.0 mgL^{-1} K.

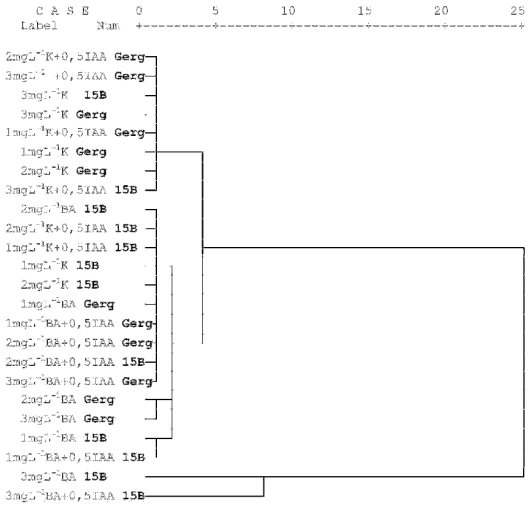


Figure 2. Hierarchical cluster analysis on the base response of two cucumber genotypes to their possibilities of callus formation, organogenesis and regeneration dipped on media cultures in cotyledon stage. Dendrogram using Average Linkage (Between Groups)

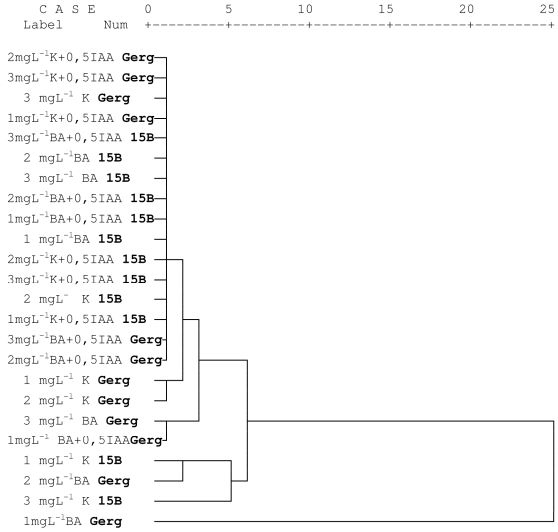


Figure 3. Hierarchical cluster analysis on the base response of two cucumber genotypes to their possibilities of callus formation dipped on media cultures in hypocotyl stage. Dendrogram using Average Linkage (Between Groups)

.

Received March 03st, 2014
Accepted July 22th, 2014

REFERENCES

ANANTHAKRISHNAN G., X. XIA, C. ELMAN, S. SINGER, H.S. PARIS, A. GAL-ON, V. GABA (2003): Shoot production in squash (*Cucurbita pepo*) by *in vitro* organogenesis. Plant Cell Reports *21*, 739-746.

AYYAPPAN V., S. NATESAN, G. ANDY, C.W. CHOI, M. MARKANDAN, K. SAMPATH (2006): Effect of ethylene inhibitors on *in vitro* shoot multiplication and their impact on ethylene production in cucumber (*Cucumis sativus* L.). Research Journal of Botany *1*, 110-117.

CURUK S., G. ANANTHAKRISHNAN, S. SINGER, X. XIA, C. ELMAN, D. NESTLE, S. CETINER, V. GABA (2003): Regeneration *in vitro* from the hypocotil of *Cucunis* species produced almost exclusively diploid shoots, and doss not require light. HortScience *38*, 105-109.

GAMBORG O.L., R. MILLER, K. OJIMA (1968): Nutrient requirements of suspension cultures of soybean root cells. Experimental Cell Research *50*, 148-151.

KOSE E., N.K. KOÇ (2003): *Agrobacterium-medited* transformation on cucumber (*Cucumis sativus* L.) and plant regeneration. Biotechnology & Biotechnological Equipment *2*, 56-62.

MOHINDDIN A.K.M., Z.C. ABDULLAH, M.K.U. CHOWDHURY, S. NAPIS (2005): Enhancement of adventitious shoot regeneration in *Cucumis sativus* L. using $AgNO_3$. Plant Tissue Culture *15*, 15-23.

MURASHIGE S., F. SKOOG (1962): A revised medium for rapid growth and bioassays wilh tabacco tisuee cultures. Phisiologiae Plantarum *15*, 473-497.

NANASATO Y., K. KONAGAYA, A. OKUZAKI, M. TSUDA, Y. TABEI (2013): Improvement of *Agrobacterium*-mediated transformation of cucumber (*Cucumis sativus* L.) by combination of vacuum infiltration and co-cultivation on filter paper wicks. Plant Biotechnology Reports *7*, 267-276.

PLADER W., S. MALEPSZY, W. BURZA, Z. RUSINOWSKI (1998): The relationship between the regeneration system and genetic variability in the cucumber (*Cucumis sativus* L.). Euphytica *103*, 9-15.

RODEVA V., S. GROZEVA, V. TODOROVA (2006): *In vitro* answer of Bulgarian pepper (*Capsicum annuum* L.) varieties. Genetika, Serbia *38*, 129-136.

SELVARAJ N., A. VASUDEVAN, M. MANICKAVASAGAM, A. GANAPATHI (2006): *In vitro* organogenesis and plant formation in cucumber. Biologia Plantarum *50*, 123-126.

SELVARAJ N., A. VASUDEVAN, M. MANICKAVASAGAM, S. KASTHURIRENGAN, A. GANAPATHI (2007): High frequency shoot regeneration from cotyledon explants of cucumber via organogenesis. Scientia Horticulturae *112*, 2-8.

SONG H., Q.F. LOU, X.D. LUO, J.N. WOLUKAU, W.P. DIAO, C.T. QIAN, J.F. CHEN (2007): Regeneration of doubled haploid plants by androgenesis of cucumber (*Cucumis sativus* L.). Plant Cell, Tissue and Organ Culture *90*, 245-254.

TODOROVA V., S. GROZEVA, V. RODEVA, S. MASHEVA (2013): Breeding evaluation of pepper lines obtained by *in vitro* anther culture. Genetika, Serbia *45,* 601-610.

UGANDHAR T., M. VENKATESHWARRLU, G. BEGUM, T. SRILATHA, K. JAGANMOHANREDDY (2011): *In Vitro* plant regeneration of Cucumber (*Cucumis sativum* (L.) from cotyledon and hypocotyl explants. Science Research Reporter *1*, 164-169.

UGANDHAR T., T. SRILATHA, M.A. IMRAN (2013): Callus induction and somatic embryogenesis from leaf explants of Cucumber (*Cucumis sativum* L.). International Journal of Integrative sciences, Innovation and Technology *2*, 29-33.

VASUDEVAN A, N. SELVARAJ, A. GANAPATHI, S. KASTHURIRENGAN, R.V. ANBAZHAGAN, M. MANICKAVASAGAM, C.W. CHOI (2008): Leucine and spermidine enhance shoot differentiation in cucumber (*Cucumis sativus* L.). In Vitro Cellular & Developmental Biology - Plant *44*, 300-306.

IN VITRO REGENERACIJA BILJAKA DVA GENOTIPA KRASTAVCA (*Cucumis sativum* L.): EFEKTI TIPA EKSPLANTA I TIPA PODLOGE GAJENJA

Stanislava GROZEVA i Nikolay VELKOV

Maritsa Institut za istraživanje povrća - Plovdiv, Plovdiv, Bugarska

Izvod

Vršena su ispitivanja efekta različitih koncentracija fitohormona na kalusogenezu I organogenezu kod dva genotipa krastavca. Utvrđeno je da brzina regeneracije zavisi od genotipa, tipa eksplanta, medijuma. U morfogenezi odgovor hipokotila je brži nego kod kotiledona. *In vitro r*egenerisani eksplanti su dobijeni kod hipokotila na podlozi sa 1.0 i 2.0 mgL^{-1} BA za genotip Gergana i 1.0 i 3.0 mgL^{-1} K –linije 15B. Indukcija kotiledona je dobijena samo kod genotipa Gergana na podlozi sa 3.0 mgL^{-1} BA i u kombinaciji sa 0.5 mgL^{-1}IAA.

Primljeno 06. III 2014.
Odobreno 22. VII. 2014.