eJBio

Electronic Journal of Biology, 2015, Vol. 11(1):17-22

In vitro regeneration in pepper (*Capsicum annuum* L.) and characterization of plant-regenerants

Stanislava Grozeva*, Velichka Todorova

Maritsa Vegetable Crops Research Institute, 32 Brezovsko Shosse Str., 4003 Plovdiv, Bulgaria.

* Corresponding author. Tel: +359 32 951227; Fax: +359 32 960177; E-mail: stanislava_grozeva@abv.bg

Abstract

The regeneration answer of hypocotyl and cotyledon explants of two pepper genotypes on MS0 medium content of macro- and micro- nutrients – ½ MS0, 1 ¼ MS0, 1 ½ MS0 1 ¾ MS0 and MS0 (control) with 3.0 mgL^{-1} BA, 0.3 mgL^{-1} IAA, 0.3 mgL^{-1} GA3 was studied. It has been found higher frequency of indirect regeneration in control culture medium while differentiation and elongation of obtained shoots has been established in culture medium variant with 1 ½ MS0 + 0.2 mgL^{-1} $AgNO_3$ and 0.3 mgL^{-1} GA3. In R_0 generation the regenerants were characterized with reduced plant height, leaf size, fruit weight and seeds per fruit. Statistical analysis of fruits morphology and productivity per plant in the next R_1 generation indicated variation between lines and compared to parents. Most lines were with shorter, but wider fruits with thicker pericarp and lower productivity per plant. For breeding purposes the complex of traits is of interest for following investigation deserve lines 3/13 and 2-1/13 from initial variety Yasen F_1. Observed differences confirm the possibility for using of somaclonal variation as a method for improving and enriching the diversity in pepper.

Keywords: Cotyledons; Hypocotyls; Macronutrients; Micronutrients; Somaclonal variation.

Abbreviations: BA - N^6-benzyladenine; IAA - Indolil-3-acetic acid; GA3 - Gibberellic acid; TDZ - Thidiazuron; $AgNO_3$ - Silver nitrate.

1. Introduction

Capsicum spp. belongs in recalcitrant plant species to *in vitro* manipulation. Several main factors affect the regeneration rate of pepper as the genotype, explant type and culture medium [1-3]. Many investigations have been conducted to optimize the regeneration process in pepper, but the major difficulties remain induction of rosettes of distorted leaves which was not developed to normal shoots [4]. Verma et al. [5] regenerated shoots from profuse rosettes after transfer for elongation to MS medium supplemented with 2.25 mgL^{-1} BA and 2.0 mgL^{-1} GA3. Joshi and Kothari [6] also used GA3 for elongation of obtained shoot buts, but combine with 30 time's increased $CuSO_4$ levels.

For plant breeding especially using somaclonal variation or genetic transformation for creation of useful genetic diversity developing of efficient plant regeneration protocol are need. The studies about gene control on *in vitro* regeneration in pepper is limited. According to Mezghani et al. [7] CDKA gene expression may be linked to dedifferentiation during adventitious organogenesis in pepper tissues cultivated *in vitro* and it can be used as a molecular marker for *in vitro* regeneration in this recalcitrant species.

Regardless of the existing difficulties and lack of effective genotype-independent protocol pepper lines different by agronomic performance and mineral content in plants and fruits were obtained [8, 9]. The aim of this research work was to study the effect of basal medium concentration of shoot induction in two pepper varieties and morphological evaluation of plant-regenerants.

2. Materials and Methods

In vitro regeneration

Seeds of two pepper varieties Yasen F_1 and Kurtovska kapia 1619 were sterilized in 5% calcium hypochlorite solution for one hour and rinsed three-times in sterile dH_2O. For germination the seeds were grown on basal medium containing macro- and micronutrients [10], vitamins [11], 3% Sucrose and 0.7% Agar (MS0). The pH of all culture medium was adjusted to 5.8 before autoclaving (121º C, 20 min).

Cotyledon (0.5 cm) and hypocotyl (1.0 cm) explants were excised from 5-7 days old *in vitro* grown seedlings and cultivated in Petri dishes on basal medium MS0 containing 3.0 mgL^{-1} BA, 0.3 mgL^{-1} IAA, 0.3 mgL^{-1} GA3, (as a control) and four variant of MS0 different in concentration of basal medium:

1. ½ MS0
2. 1 ¼ MS0
3. 1 ½ MS0
4. 1 ¾ MS0

After 40 days the explants were transferred to the same culture medium without BA and IAA, but supplemented with 0.2 mgL^{-1} $AgNO_3$ and 0.3 mgL^{-1} GA3. Cultures were incubated in a growth chamber:

temperature 26ºC±1ºC, a photosynthetic proton flux density (PPFD) of 200 µmol m^{-2} s^{-1} and 14/10 h photoperiod and sub-cultured at intervals of 20 days to the same medium variants. Well-developed shoots (2-3 cm) were isolated from explants and transferred on rooting medium consisting of ½ MS0 and 0.1 mgL^{-1} IAA.

The experimental design for regeneration was: three replication with 20 explants of each, twice, 120 explants per treatment – culture medium variant, explant type and genotype. The callusogenesis, organogenesis and regeneration frequency (% explants with response) and number of regenerants per explant (compared to reacted with organogenesis explants) were examined for a period of 90 days.

Plant adaptation and acclimatization

Regenerants 4-5 cm in height were extracted from the culture medium, the roots were rinsed with running water and planted in 0.5 L pots in mixture peat moss and perlite in the ratio 1:3 (v/v) for 10-12 days. Successfully adapted plants were transferred in 5 L containers mixture peat moss and perlite in the ratio 1:1 (v/v) and grown in glasshouse. As a control was used *in vitro* germinated and adapted plants from initial genotypes.

Evaluation on morphological characters

The following traits between plants derived both from *in vitro* culture and from seeds from R_0 progeny were recorded: plant height (cm); leaf blade – length and width (cm), leaf colour, pollen fertility (squashing in 4% acetocarmine and glycerin (1:1). After self-pollinated 9 R_1 lines were compared by fruit weight (g), length (cm) and width (cm), usable part of the fruit (g) and pericarp thickness (mm), productivity per plant (g),number of fruits per plant and seeds per fruit (number). These traits were evaluated on five plants per replication and three fruits per plants.

The plants for R_1 were grown under field conditions in two replications with 15 plants of each line and initial variety on furrow surface by 70/15 cm scheme. The plants were grown according to the adopted technology for mid-early field production.

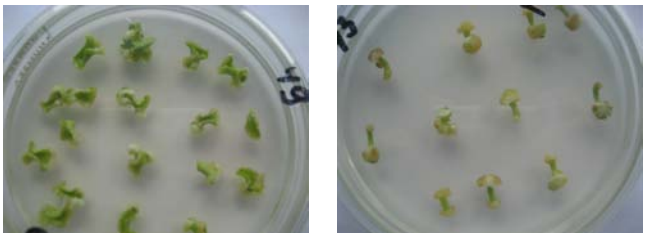
Statistical data analysis

The results of regeneration frequency and morphology traits were are given as means ± standard deviation (SD). Data were subjected to Duncan's Multiple Range Test to evaluate the statistical significance among the means. Three-way analysis of variance for influence of variation factors on the regeneration was studied.

3. Results and Discussion

***In vitro* regeneration**

Hypocotyl and cotyledon segments showed induction of callus of the cut edges after 10 day of cultivation in all culture medium. Callus morphology in cotyledons was friable and whitish with intensive growth. In hypocotyls callus was compact, white and hard with slower growth (Figure 1a, b). Initial formation of organogenic structures was about 15-20 days of cultured. Organogenic frequency in cotyledons varied from 3.3% (medium variant 4) to 100% (control medium) in variety Yasen F_1 and from 20% (medium variant 4) to 100% (control medium) in variety Kurtovska kapia 1619 (Figure 2). On the other hand organogenic reaction in hypocotyls was registered only in Yasen F_1 in control culture medium and variant 3. However, after 2 or 3 subcultures of the fresh medium the explants were covered by more callus tissue and subsequent differentiation and elongation of shoot regeneration decreased (Figure 1c, d). Verma et al. [5] also observed more shoots buds during initial stages of callus proliferation and decreased shoot regeneration during later stages of callus grown.



a. Cotyledons b. Hypocotyls

c. Cotyledons d. Hypocotyls

Figure 1. Callusogenesis. (**a, b**): 10-12 days of cultured; (**c, d**): 40 days of cultured.

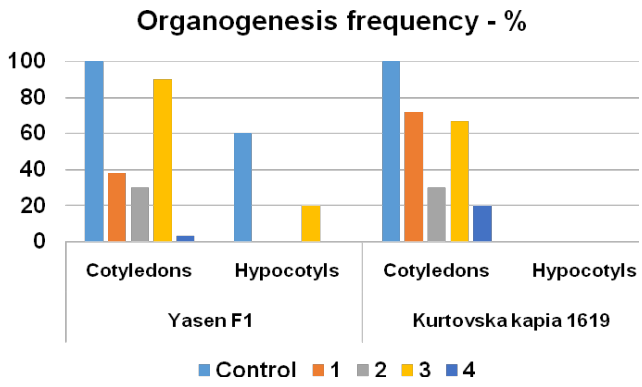


Figure 2. Organogenic response.

Differences in the regeneration potential were observed depending on the Genotype (Factor A), Explant type (Factor B) and Culture medium (Factor C) (Figure 3). Statistical data showed the strongest

influence of Culture medium (38.13%) followed by interaction Genotype x Culture medium (21.57%), Explant type x Culture medium (14.95%) and Culture medium (14.00%). Significantly lower were effects of Genotype (3.16%) and interaction Genotype x Explant type x Culture medium (2.61%).

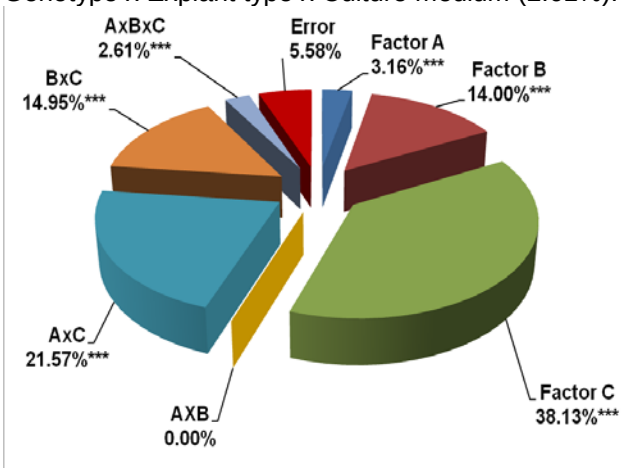


Figure 3. Three-way analysis of variance and power of influence of variation factors on the regeneration depend on genotype, explant types and culture medium

In both studied pepper genotypes the regeneration frequency varied from 0.0% to 48.3% (Table 1). The highest percentage of regenerated cotyledon explants in variety Yasen F_1 was achieved in control medium variant (48.3%), while in variety Kurtovska kapia 1619 in variant 3 (33.3%) contained 1 ½ basal MS0 medium. Reaction with lower frequency was registered in culture medium variant 2 with 1 ¼ strength. When culture medium contained ½ and 1 ¾ strength (variants 1 and 4 respectively) regeneration process was absent. Regeneration in hypocotyls was obtained only in variety Yasen F_1 under control medium.

After transfer of explants on elongation medium the highest number of regenerants per cotyledon explant in both genotypes was on 1 ½ (variant 3) followed by control medium (Figure 4a, b). Hypocotyls reacted only on control medium in Yasen F_1 (Table 2). To induce regeneration in pepper from different explants combinations of BA or TDZ with IAA are applied, but the main difficult is elongation of obtained shoot buds [1, 12]. Cytokinins stimulate shoot proliferation and inhibit their elongation. To overcome the problem with shoot elongation culture medium without cytokinins was used [13, 14]. Pishbin et al. [3] reported to better shoot elongation in culture medium contained low concentration of BA (0.1 mgL^{-1}) and GA3 (0.5 mgL^{-1}). In contrast fair elongation Verma et al. [5] obtained after shoots transfer on 2.25 mgL^{-1} BA and 2.0 mgL^{-1} GA3. The differences observed in this study may be due to the distinctions in concentration of macro- and microelements of basal medium. Increasing of concentration of basal elements stimulated shoot elongation and differentiation to plants but have no such effect on regeneration. In cherry Ruzic et al. [15] reported for the best multiplication on MS 2× and MS media which they have associated with the highest N and P content.

Table 1. Regeneration frequency in hypocotyls and cotyledons of two pepper varieties.

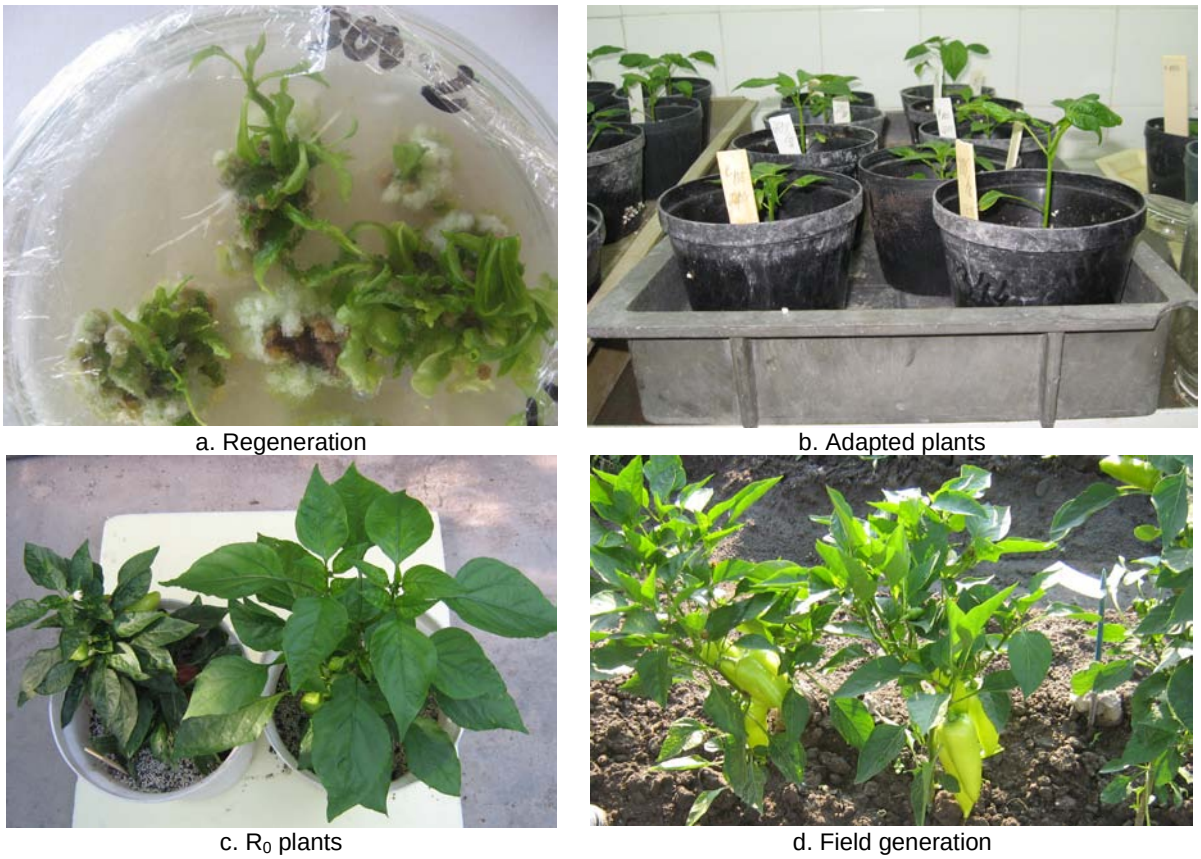
Culture medium	**Explant types**							
	cotyledon		hypocotyl		cotyledon		hypocotyl	
	%	SD	%	SD	%r	SD	%	SD
variety	**Yasen F_1**				**Kurtovska kapia 1619**			
Control	48.3a	±2.89	23.3a	±5.77	13.3b	±7.64	0.0	±0.00
1	0.0d	±0.00	0.0b	±0.00	0.0c	±0.00	0.0	±0.00
2	8.3c	±2.89	0.0b	±0.00	3.3bc	±5.77	0.0	±0.00
3	18.3b	±5.77	0.0b	±0.00	33.3a	±11.55	0.0	±0.00
4	0.0d	±0.00	0.0b	±0.00	0.0c	±0.00	0.0	±0.00

Note: Values are means ± standard deviation. Values in columns followed by different letters are significantly different at a,b,c….p≤0.05 Duncan's Multiple Range Test (n=6).

Table 2. Effect of culture medium of shoot elongation.

Culture medium	**Explant types**							
	cotyledon		hypocotyl		cotyledon		hypocotyl	
	number	SD	number	SD	number	SD	number	SD
variety	**Yasen F_1**				**Kurtovska kapia 1619**			
Control	1.12b	±0.22	0.67a	±0.10	0.35b	±0.18	0.00	±0.00
1	0.00d	±0.00	0.00b	±0.00	0.00c	±0.00	0.00	±0.00
2	0.67c	±0.29	0.00b	±0.00	0.11bc	±0.19	0.00	±0.00
3	1.77ab	±0.10	0.00b	±0.00	1.18a	±0.23	0.00	±0.00
4	0.00d	±0.00	0.00b	±0.00	0.00c	±0.00	0.00	±0.00

Note: Values are means ± standard deviation. Values in columns followed by different letters are significantly different at a,b,c….p≤0.05 Duncan's Multiple Range Test (n=6).



a. Regeneration b. Adapted plants

c. R_0 plants d. Field generation

Figure 4. Somaclonal variation in pepper.

The morphological characterization of R_0 *in vitro* regenerants and seed-derived plants from Yasen F_1 and Kurtovska kapia 1619 is given in Table 3. Regenerants were with reduced plant height, leaf size, fruit weight and seeds per fruit compared to the seed-derived plants (Figure 4c). Pollen fertility between regenerants and seed-derived plants were 70.1% and 91.7% in Yasen F_1 and 68.9% and 87.3% in Kurtovska kapia 1619, respectively. The regenerants from variety Kurtovska kapia 1619 were distinguished with green leaves in contrast to dark green colour in control plants. After self-pollination of regenerants seeds from the next investigation were produced from 9 plants – 6 originated from variety Yasen F_1 and 3 – Kurtovska kapia 1619.

Table 3. Study of morphological characterization of *in vitro* and seed-derived plants in R_0.

Genotype	**Yasen F_1**		**Kurtovska kapia 1619**	
Traits	***In vitro***	**Seed**	***In vitro***	**Seed**
Plant height (cm)	39.0	53.0	13.3	40.0
Leaf length (cm)	12.6	23.7	6.8	20.6
Leaf width (cm)	5.5	6.8	3.4	6.5
Leaf color	green	green	green	dark green
Fruit weight (g)	35.3	63.7	22.9	70.8
Seeds per fruit	47	157	44	124
Pollen fertility (%)	70.1	91.7	68.6	87.3

The results from the characterization of the R_1 lines of pepper showed that regenerants originating from variety Kurtovska kapia 1619 were distinguished with decreased values of the studied traits. Only the characters fruit width and pericarp thickness were with values close to the control plants (Table 4). However diversity among the lines obtained of variety Yasen F_1 was higher. The average fruit weight in regenerants varied from 46.69 g to 95.68 g, while fruit length and width varied in narrow margins – 9.23/4.83 cm to 12.37/5.80 cm, respectively. The usable part of the fruit varied wider than the pericarp thickness. The variation interval was from 30.92 g to 68.04 g for usable part of the fruit and 3.67 mm to 4.67 mm – pericarp thickness. Only fruits from lines 2/13 and 5/13 were with the lowest seeds per fruits (30 and 57 number, respectively). Significant differences between lines were established by productivity per plant (from 390 g to 1130 g) and number of fruits per plant (from 7 to 17). It has been found that most regenerants from variety Yasen F_1 were with shorter, but wider fruits with thicker pericarp and lower productivity per plant than parent. Only line 3/13 was with productivity per plant, close to the control. For breeding purposes by complex of traits interest for following investigation are lines 3/13 and 2-1/13 from variety Yasen F_1 (Figure 4d). On the other hand studies in other lines also need to continue because they may have other useful features, such as disease or pest resistance, fruit quality, etc.

which contribute to enrich the population of pepper with new gene combination.

Table 4. Field experiments of R_1 lines.

Genotype	Fruit Weight (g)	Fruit length (cm)	Fruit width (cm)	Usable part of the fruit (g)	Pericarp thickness (mm)	Seeds per fruit (No.)	Productivity per plant (g)	Number of fruits per plant
1/13	53.22bc	10.27bc	4.43bc	38.61b	4.67a	150b	980	17
SD	7.74	1.75	0.29	8.16	0.58	44.64		
2/13	50.95bc	9.67bc	4.63bc	36.79b	3.67ab	30d	660	11
SD	0.04	1.21	0.55	2.33	0.58	21.07		
3/13	68.86abc	9.33bc	5.30ab	50.37ab	4.00a	265a	1130	16
SD	30.43	2.37	0.95	25.26	1.00	7.00		
5/13	42.69c	9.23c	4.33c	30.92b	4.00a	57cd	500	11
SD	4.59	0.68	0.25	3.21	0.00	46.92		
7/13	67.12bc	11.50abc	4.87bc	52.45ab	3.67ab	114bc	390	7
SD	9.80	2.36	0.50	9.82	0.58	50.85		
2-1/13	95.68a	12.37ab	5.80a	68.04a	4.67a	265a	800	12
SD	13.78	1.14	0.20	11.39	0.29	25.01		
Yasen F_1	**76.89ab**	**13.47a**	**4.90abc**	**48.80ab**	**2.67b**	**220a**	**1200**	**14**
SD	17.59	0.06	0.20	0.58	0.58	11.24		
KK4/13	32.28c	5.77c	4.43b	21.45c	4.00ab	136c	900	20
SD	0.99	0.85	0.32	1.55	1.00	44.84		
KK2-6/13	45.28b	8.37b	4.70ab	31.73bc	3.67c	166bc	250	7
SD	4.26	0.55	0.35	4.75	0.58	29.14		
KK2-8/13	57.16ab	8.23b	5.30a	38.80b	4.67a	180b	320	6
SD	6.59	0.59	0.10	3.17	0.58	27.87		
K. kapia 1619	**63.53a**	**11.31a**	**4.58ab**	**50.04a**	**4.05ab**	**235a**	**1020**	**16**
SD	3.67	0.42	0.11	1.25	0.23	20.36		

Note: Values in columns followed by different letters are significantly different at a,b,c….p≤0.05 Duncan's Multiple Range Test.

In *Solanaceae* family somaclonal variation as a method leading to creation of suitable genetic diversity and its involving in the breeding process is applied with success in tomato, potato and tobacco. Pepper belongs to recalcitrant species with respect to its *in vitro* regeneration ability. Little is known about the morphological and genetic variations in somaclones of pepper because of more studies are directed to overcome the difficulties with regeneration process [16]. The source of somaclonal variation depends on genotype, explant type and culture conditions especially basal culture medium, concentration and combination of plant growth regulators etc. [17]. Clear information about genetic mechanisms controlling organogenesis is still limited. Positive correlation between CDKA gene expression and the proliferation stage of pepper cotyledon tissue culture in vitro was proved [7]. The authors concluded that CDKA expression seemed to be a useful molecular marker for the cell dedifferentiation phase. A better understanding of recalcitrant nature will provide an opportunity to

identify the regulatory genes of pepper morphogenesis and process [16]. Morphology characterization and random amplified polymorphic DNA analysis are used to evaluate the somaclonal variation in pepper [5, 16]. In R_0 generation variations in growth habit, stem and flower colour, pollen fertility, fruit morphology, productivity per plant etc. were observed [8]. The studies of the inheritance of changes traits in the R_1 generation were also conducted [18]. Pepper lines with early flowering, increase of yield and yield components [18, 19], different in fruits colour and shape [19], quality traits and mineral content in the fruits [9] were developed on the base of somaclonal variation.

4. Conclusions

In vitro organogenesis and regeneration in pepper is affected by genotype, explants and concentration of basal medium. Cotyledons are more susceptible to regeneration than hypocotyls. Better regeneration was achieved in control medium. The best elongation of obtained shoots was registered in culture medium variant with 1 ½ MS0 supplimented with 0.2 mgL^{-1} $AgNO_3$ and 0.3 mgL^{-1} GA3.

Morphological differences in R_0 were observed between *in vitro* regenerants and seed-derived plants. Somaclones in next R_1 generation showed same favorable changes like increase of fruit weight, width and pericarp thickness. The obtained variability in both studied genotypes indicates the possibility of improvement of pepper through somaclonal variation.

References

[1] Grozeva S., Rodeva V., Todorova V. (2012) *In vitro* shoot organogenesis in Bulgarian sweet pepper (*Capsicum annuum* L.) varieties. *EJBio*, **8(3)**: 39-44.

[2] Kumar R.V., Sharma V.K., Chattopadhyay B., Chakraborty S. (2012) An improved plant regeneration and *Agrobacterium* – mediated transformation of red pepper (*Capsicum annuum* L.). *Physiol Mol Biol Plants*, **18(4)**: 357-364.

[3] Pishbin N., Mousavi A., Kalatejari S., Shariatpanahi M., Behrooznam Jahromi B. (2014) The effect of plant growth regulators and different types of explants on *in vitro* regeneration of sweet pepper (*Capsicum annuum* L.). *Int. J. Biosci*., **5**: 139-146.

[4] Orlinska M., Nowaczyk P. (2015) *In vitro* plant regeneration of 4 *Capsicum* spp. genotypes using different explant types. *Turk. J. Biol*., **39**: 60-68.

[5] Verma S., Dhiman K., Srivastava D.K. (2013) Efficiency *in vitro* regeneration from cotyledon explants in bell pepper (*Capsicum annuum* L. cv. California wonder). *Intl. J. Adv. Biotec. Res.*, **4(3):** 391-396.

[6] Joshi A., Kothari S.L. (2007) High copper levels in the medium improves differentiation and elongation from cultured cotyledons of *Capsicum annuum* L. *Plant Cell Tiss. Org. Cult*., **88**: 127-133.

[7] Mezghani N., Gargouri-Bouzid R., Laliberté J., Elloumi N., Tarchoun N., Jemmali A. (2014) Characterization of CDKA gene expressed during *in vitro* regeneration from pepper (*Capsicum annuum* L.) cotyledon explants. *IJAAR*, **5(5)**: 29-39.

[8] Valadez-Bustos M.G., Aguado-Santacrus G.A., Carrillo-Castaneda G., Aguilar-Rincon V.H., Espitia-Rangel E., Montes-Hernandez S., Robledo-Raz A. (2009) *In vitro* propagation and agronomic performance of regenerated chilli pepper (*Capsicum* spp.) plants from commercially improvement genotypes. *In Vitro Cell. Dev. Biol. Plant.*, **45**: 650-658.

[9] Koleva Gudeva L., Veselinovska S. (2011) Some physiological characteristics of pepper (*Capsicum annuum* L.) produced *in vitro*. *EJBio*, **7(1)**: 1-5.

[10] Murashige T., Skoog F. (1962) A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol. Plant*., **15**: 473-497.

[11] Gamborg O.L., Miller R.A., Ojima K. (1968) Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res*., **50**: 148-151.

[12] Aniel Kumar O., Rupavati T., Subba Tata S. (2012) Adventitious shoot bud induction in chili pepper (*Capsicum annuum* L. cv. X-235). *I.J.S.N*., **3(1)**: 192-196.

[13] Ahmad N., Siddique I., Anis M. (2006) Improved plant regeneration *in Capsicum annuum* L. from nodal segments. *Biol. Plant*., **50**: 701-704.

[14] Rizwan M., Sharma R., Soni P., Gupta N.K., Singh G. (2013) Regeneration protocol for chilli (*Capsicum annuum* L.) variety Mathania*. J. Cell Tissue Research*, **13(1)**: 3513-3517.

[15] Ruzic D., Saric M., Cerovic R., Culofic L. (2000) Relationship between the concentration of microelements their uptake and multiplication of cherry rootstock Gisela 5 *in vitro*. *Plant Cell Tiss. Org. Cult.*, **63**: 9-14.

[16] Santana-Buzzy N., Bello-Bello J.J., Iglesias-Andreu L., ZUniga-Aguilar J.J., Canto-Flick A., Aviles-Vilias S.A., Lecona-Guzman C.A., Solis-Marroquin D., Gomez-Uc E., Balam-Uc E., Arcos-Ortega G.F., Mijangos-Cortes J.O. (2012) Tissue culture of *Capsicum* species.In *Peppers, Botany, Production and Uses* (Vincent M. Russo ed.). CAB International, pp. 72-86.

[17] Brar D.S., Jain S.M. (1998) Somaclonal variation: mechanism and applications in crop improvement. In: *Current Plant Science and Biotechnology in Agriculture* (Jain S.M., Brar D.S., Ahloowalia B.S. eds). Kluwer Academic Publishers, pp. 15-37.

[18] Hossain M., Konisho K., Minami M., Nemoto K. (2003) Somaclonal variation of regenerated plants in chili pepper (*Capsicum annuum* L.). *Euphytica*, **130**: 233-239.

[19] Sargsyan G.J., Vardanian I.V., Harutynyan, Z.E. (2014) Usage of callus culture in selection of pepper and tomato. *Acta Hort*., (ISHS) **1033**: 77-84.