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In vitro Plant Regeneration of Two Cucumis melo L. Genotypes Using Different Explant Types and Culture Medium

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Abstract. Development of an efficient *in vitro* plant regeneration system plays an important role for applying of biotechnological approaches for crop improvement. Therefore, the effect of added of BAP in germination phase on organogenesis of cotyledon and hypocotyl explants of two melon lines was investigated. The seeds germination medium contained three different concentrations of BAP (0.5, 1.0 and 1.5 mg L^{-1}). The rate of plant regeneration was found to depend on genotype, explant type and culture medium. The cotyledons were more effective as explants for organogenesis and subsequent plant elongation than hypocotyls. Combination of 1.0 mg L^{-1} of BAP in germination medium and 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} IAA in next regeneration medium gives the better regeneration answer in two explant types of line 11/9, while in line AGY the most effective was the combination of 1.5 mg L^{-1} of BAP and 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} IAA. The experimental results indicated that pre-treatment with cytokinin BAP (1.0 mg L^{-1}) in germination stage stimulates regeneration process in cotyledons and hypocotyls of melon line 11/9.

Key words: melon, cotyledons, hypocotyls, pre-treatment, cytokinin.

Introduction

Melon is one of the most important crops from Cucurbitaceous family which is described with great diversity of groups (ROBINSON & DECKER-WALTERS, 1997). Three varieties of *Cucumis melo* L. (var. *cantalupensis*, var. *reticulatus* and var. *inodorus*) are involved in breeding program in the Maritsa Vegetable Crops Research Institute – Plovdiv, Bulgaria (VELKOV & PETKOVA, 2014). Traditional breeding methods in melon have led to a considerable varietal improvement (NUÑEZ-PALENIUS *et al*, 2008). However, strong sexual incompatibility barriers at the interspecific and intergeneric levels have restricted the use of that genetic potential to develop new and enhanced melon cultivars (NIEMIROWICZSZCZYTT & KUBICKI, 1979; ROBINSON & DECKER-WALTERS, 1999). Somaclonal variation is another important source of genetic diversity to obtain new hereditary variations useful in plant breeding (JAIN, 2001; LESTARI, 2006).

In vitro plant regeneration in melon has been achieved via direct and indirect organogenesis by use of diverse explant types: hypocotyls, cotyledons, leaves, cotyledonary nodes and petioles (CURUK *et al.*, 2003; SOUZA *et al.*, 2006; ZHANG *et al.*,

2011; NADERI *et al.*, 2013; IVANOVA *et al.*, 2017). Shoot induction and regeneration have been obtained in culture medium supplemented with different combinations and concentrations of growth regulators (MELARA & ARIAS, 2009; MENDI *et al.*, 2010a; AWATEF & MOHAMED, 2013; NADERI *et al.*, 2016). Beneficial effects of 6-Benzylaminopurine (BAP) or kinetin in combination with Indolil-3-acetic acid (IAA) on shoot induction have been observed in melon by several authors (LIBORIO *et al.*, 2001; CHOI *et al.*, 2012; SEBASTIANI & FICCADENTI, 2013). However, auxins are not always a prerequisite to achieve that goal, and cytokinins alone are able to induce bud formation (KENG & HOONG, 2005; REN *et al.*, 2013). NADERI & MAHMOUDI (2017) reported that the application of 1.5 mg L^{-1} BAP plus 250 mg L^{-1} cefotaxim and 1 mg L^{-1} BAP with 1000 mg L^{-1} cefotaxim formed the most efficient media for plant regeneration. On the other hand, MENDI *et al.*, (2010a) obtained more regenerants per explant on medium supplemented with combination of 0.5 mgL^{-1} BAP and 0.5 mg L^{-1} IAA (88%), compared to the use of 1.0 mg L^{-1} BAP (75%) alone. KISS-BÁBA *et al.*, (2010) also underlined the positive effect of IAA adding to BAP on shoot induction and regeneration in melon. However, some of the genotypes are recalcitrant and do not react with normal shoot-buts and plantlets formation (STIPP *et al.*, 2001).

Several experiments have been carried out to optimize culture conditions and to overcome the difficulties for shoot elongation and differentiation. Pre-treatment with phytohormones, explant source and position are used, but the success of these trails is with different effect (KISS-BABA *et al.*, 2010; DO AMARAL *et al.*, 2014). According to CHOVELON *et al.* (2011) environmental and hormonal requirement for melon regeneration are poorly understood, which makes difficult development of routine procedure for plant regeneration. Appropriate combining of explant type and culture conditions could significantly increased the regeneration frequency of melon (GUIS *et al.*, 1998).

The aim of this experimental work was to study regeneration potential of different explant types and culture medium of two melon lines.

Materials and Methods

Plant material

The experimental work was carried out during the period 2016-2017 with two melon lines 11/9 and AGY, which is part of the Maritsa Vegetable Crops Research Institute's collection. Melon line 11/9 belongs to var. *cantalupensis* which is described with monoecious type of flowering, male sterility (ms-4), fruits possess elliptical shape, yellow ground colour of skin, orange colour of the flesh. Breeding line AGY belongs to var. *reticulatus* (Ananas type) which is described with gynoecious type of flowering, fruits possess oval shape, netted, yellow ground colour of skin, white colour of the flesh. Both lines are elite that have been inbred five times.

Explant types and treatment

Seeds of the melon lines were surface sterilized in 5% calcium hypochlorite solution for 1 hour and rinsed three-times in sterile dH_2O. For germination the seeds were sown on three variants of basal culture medium containing macro- and micronutrients by MURASHIGE & SKOOG (1962), vitamins by GAMBORG *et al.* (1968), 30 g L^{-1} sucrose and 0.7% agar (MS0), differ by concentration of cytokinin 6-Benzylaminopurine (BAP) (0.5, 1.0 and 1.5 mg L^{-1}), named MS1, MS2 and MS3, respectively. The pH of the medium was adjusted to 5.8 before autoclaving. All culture media were autoclaved at 121 °C for 20 minutes.

Explants of cotyledons (0.5 cm^2) and hypocotyls (1.0 cm) were excised from 5-7 days old *in vitro* grown seedlings and cultivated in Petri dishes on MS0 medium. The next three combinations of BAP and IAA (Indolil-3-acetic acid) were studied:

1. 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} IAA
2. 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} IAA
3. 1.5 mg L^{-1} BAP + 0.5 mg L^{-1} IAA

When the regenerants were 2-3 cm long, they were transferred into 250 ml glass jars containing 25 ml ½ MS0 medium (half strength medium), 30 g L^{-1} Sucrose, 0.7% Agar (rooting medium). For adaptation the plantlets 5-6 cm long were transferred in mixture of perlite:peat (1:1) (v/v), for a period of 10-12 days. Subsequently, the plantlets were transplanted to 5 L plastic pots with peat moss and perlite in ratio 1:1 (v/v) in greenhouse conditions.

The explants and regenerants were incubated in growth chamber at 25°C ± 1°C temperature, a photosynthetic proton flux density (PPFD) of 200 µmol m^{-2} s^{-1}, 16/8 h photoperiod and subcultured at intervals of 20 days to the same medium variants.

The experiment was carried out in three replications with 20 explants in each for the different genotypes, medium variants and explant types. The callusogenesis, organogenesis, regeneration frequency (% explants with regeneration) and number of regenerants per explant were examined for a period of 90 days.

Statistical analysis

All data were statistically analyzed using the SPSS (SPSS INC., CHICAGO, USA, 2008) and Excel (MICROSOFT CO., 2016). Four-way analysis of variance and LSD test were performed at P = 0.05 on each of the significant variables measured.

Results and Discussion

Melon cotyledon and hypocotyl explants from two studied lines reacted with callusogenesis in all studied medium variants. Depending of the explant type the callus morphology respond was different. In cotyledons callus was friable and whitish with leaf structures and elongated shoots, while in hypocotyls callus was transparent and watery with sporadic regeneration answer. Regeneration through callus phase is a precondition for induction of genetically stable changes in obtained plant-regenerants with higher frequency (NUNEZ-PALENIUS *et al.*, 2008).

The frequency of organogenesis in the studied melon lines depending on explant types and culture medium variants which varied from 0.0% to 100%. These results corresponded with other studies conducted under different genotypes and concentration of plant-growth regulators (CHEE, 1991; CURUK *et al.*, 2003; TEKDAL & CETINER, 2013). This could be explained by lack of correlations between explants type and components of cultural media and empiric investigations are reasonable.

Organogenic response was better expressed in cotyledons than hypocotyls. Organogenic process in hypocotyls of line 11/9 cultivated in culture medium variant MS3 containing 1.5 mg L^{-1} BAP was not established (Fig. 1).

Induction of organogenesis in explants of line AGY was lower than line 11/9. The response of cotyledons of line AGY cultivated in germination medium MS2 was not observed whereas in the other medium variants it ranged within 15.0% – 100%. In hypocotyls organogenic structures was observed only in control germination medium (MS0) and combination MS3 + 2 variant of regeneration medium (Fig. 2). The genotype is the most important factor determining organogenic and regeneration potential. FICCADENTI & ROTINO (1995) have observed different reaction among the eleven cultivars belonging to the *reticulatus* and *inodorus* genotypes. The authors have found that *C. melo* var *inodorus* had a uniformly high regeneration rate whereas *reticulatus* varieties exhibited wide differences in their organogenesis. MOLINA & NUEZ (1995) have found genotypic variability of the *in vitro* answer in individual melon seeds. GALPERIN *et al.* (2003) has screened 30 different commercial melon cultivars for shoot *de novo* regeneration, but only one BU-21, had profuse regeneration of multiple shoots. The effect of the genotype on regeneration process in melon was demonstrated in

current study, but other factors as explant types, hormonal regulation under *in vitro* condition enhance or limit melon regeneration.

Appropriate choice of the explant significantly influences the morphogenic ability. In melons, younger, smaller leaves and very young cotyledons were found to be most responsive (SOUZA *et al.*, 2006). Differences observed in organogenesis in the both explant types may be due to also the influence of endogenous growth regulators and combination of these added in culture medium. For example, LESHEM (1989) showed shoot formation mainly proceeds from basal edge of the cotyledons and suggesting that this probably due to the accumulation of an endogenous growth regulator such as auxins. TORELLI *et al.* (2004) reported that the regeneration in hypocotyls of tomato is position-dependent and associated with the movement and different concentration of hormones in the tissues.

The regeneration process of melon is limited by many factors. The main four factors that were studied Germination medium (Factor A), Regeneration medium (Factor B), Genotype (Factor C) and Expant type (Factor D) have proven influences on regeneration answer of melon (Table 1). Statistical data indicated that effect of explant type (η=32.65%) dominated over other factors, followed by Genotype (η=9.37%). Regeneration process strongly influenced also of interaction between Germination medium and Explant type (η=13.30%). Statistical significant differences were not demonstrated in interaction of factors B x D and B x C x D, which shows the important role of pre-treatment on regeneration in melon and specifically requirement of the genotype established by other authors.

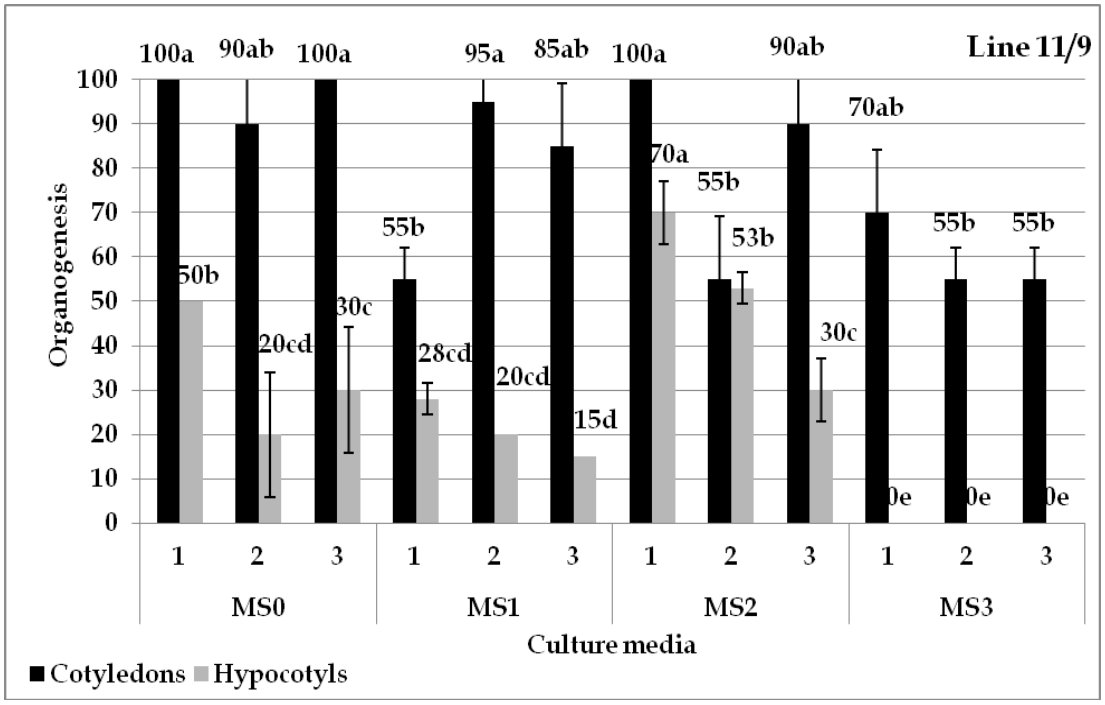

Fig. 1. Organogenic reaction in cotyledons and hypocotyls of line 11/9 in different culture medium variants. Values in columns followed by different letters are significantly different at P≤0.05 based on LSD Test (n=3).

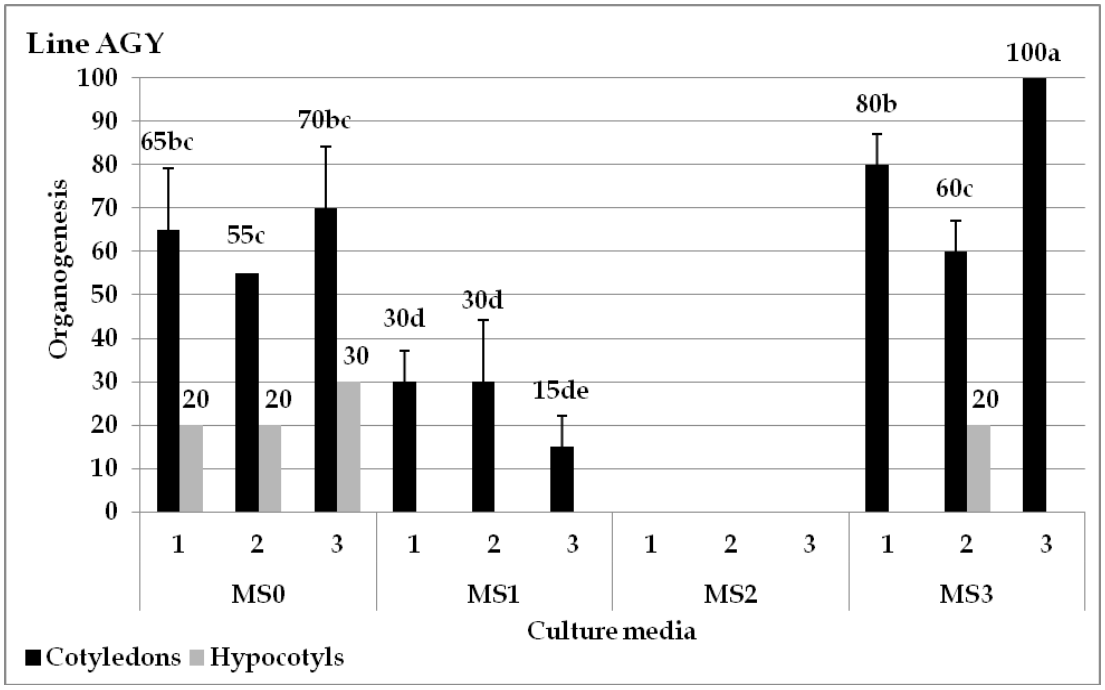

Fig. 2. Organogenic reaction in cotyledons and hypocotyls of AGY in different culture medium variants. Values in columns followed by different letters are significantly different at P≤0.05 based on LSD Test (n=3).

Table 1. Four-way analysis of variance and power of influence of variation factors on the regeneration depend on germination medium, regeneration medium, line and explant type. *Legend:* * - P ≤ 0,05; ** - P ≤ 0,01; *** - P ≤ 0,001; n.s. – no significant.

Source	Type III Sum of Squares	df	Mean Square	Power of influence (η%)	Sig.
A (Germination medium)	6269.531	3	2089.844	7.53	***
B (Regeneration medium)	916.146	2	458.073	1.10	**
C (Genotype)	7794.010	1	7794.010	9.37	***
D (Explant type)	27169.010	1	27169.010	32.65	***
A * B	2160.938	6	360.156	2.60	**
A * C	11065.365	3	3688.455	13.30	***
B * C	1109.896	2	554.948	1.33	**
A * B * C	5833.854	6	972.309	7.01	***
A * D	3840.365	3	1280.122	4.61	***
B * D	109.896	2	54.948	0.13	n.s.
A * B * D	2558.854	6	426.476	3.07	***
C * D	4469.010	1	4469.010	5.37	***
A * C * D	2690.365	3	896.788	3.23	***
B * C * D	178.646	2	89.323	0.21	n.s.
A * B * C * D	2990.104	6	498.351	3.59	***
Error	4062.500	48	84.635	4.88	
Total	83218.490	95			

There are a few studies of pre-treatment with cytokinin BAP in germination stage on regeneration in melon. DO AMARAL *et al.* (2014) established a positive influence on the regeneration rate after adding of BAP in germination stage of recalcitrant cultivar 'Gaucho'. Histological analyses confirmed the development of more adventitious shoots when explants were cut of plants germinated of culture medium with low BAP concentration. Therefore, the addition of BAP in the culture medium during the phases of seed germination and regeneration must have affected the internal hormonal balance of the explants (WANG *et al.*, 2011). In contrast, PINHO *et al.* (2010) in the same cultivar have achieved 29.66% regeneration frequency, even when the explants were removed from the plants grown on medium supplemented with BAP. According to KINTZIOS *et al.* (2002) the positive effect of pre-treatment before regeneration stage with growth regulators in embryogenesis of melon may be help cells to enter organogenic differentiation pathways.

Data presented in Table 2 shows that adding of BAP in germination medium stimulates regeneration answer of studied melon lines. However, increasing of BAP concentration during germination and higher levels of this hormone in regeneration medium leads to reduction of shoot development. The highest percentage of cotyledons and hypocotyls reacted with regeneration from line 11/9 was obtained in combination of germination culture medium variants MS2 and regeneration medium variant 1 (100% and 50%, respectively), followed by MS0 + 1 (85% and 40%, respectively). In these combinations the number of regenerants per explant was high (1.65 and 1.50, respectively), but the highest regenerants was obtained in combination MS2 + 2 (1.75) (Fig. 3 a, b, c). The similar results were reported by DO AMARAL *et al.* (2014). In line AGY regeneration process was registered only in cotyledons in eight of the twelve studied culture medium combinations. The highest value of regenerated explants was measured in combination of culture medium MS3 and 1 (75%), followed by MS0 + 2 (50%).

In vitro organogenesis was stimulated usually of combination of cytokinin and auxin in a certain ration depending on the genotype. Better regeneration answer is observed when explants were transferred on regeneration medium containing lower BAP (0.5 and 1.0 mg L^{-1}) and IAA (0.5 mg L^{-1}) concentrations. Higher levels of BAP decrease regeneration rate. Ficcadeni & Rotino (1995) have concluded that BAP was able to induce shoot formation, but the combination with Abscisic acid significantly increased the number of shoots per explant. According to Keng & Hoong (2005) addition of auxin in regeneration medium was not contributed multiple shoot formation, but was induced formation of friable callus. These results confirmed the data obtained by REN *et al.* (2013). On the other hand, the highest shoot formation was obtained from culture medium containing 0.5 mg L^{-1} BAP and 0.5 mg L^{-1} IAA (88%), while the lowest one – 1.0 mg L^{-1} BAP (75%) alone or in combination with 0.1 mg L^{-1} IAA (60%) (MENDI *et al.*, 2010a). The influence of combination of BAP with auxins (IAA, NAA, 2,4-D) on regeneration in melon was reported by many authors and the effect depends on genotype, explant type and additional culture conditions (MENDI *et al.*, 2010b; ZHANG *et al.*, 2011; IVANOVA *et al.*, 2017). The regenerants from line 11/9 (20 plants) and line AGY (7 plants) were successful adapted and acclimatized (Fig. 3 d, e, f). Significant differences in morphology characters between obtained regenerants and initial lines were not observed. During the growth season, the plants were self-pollinated and seeds were received for subsequent studies.

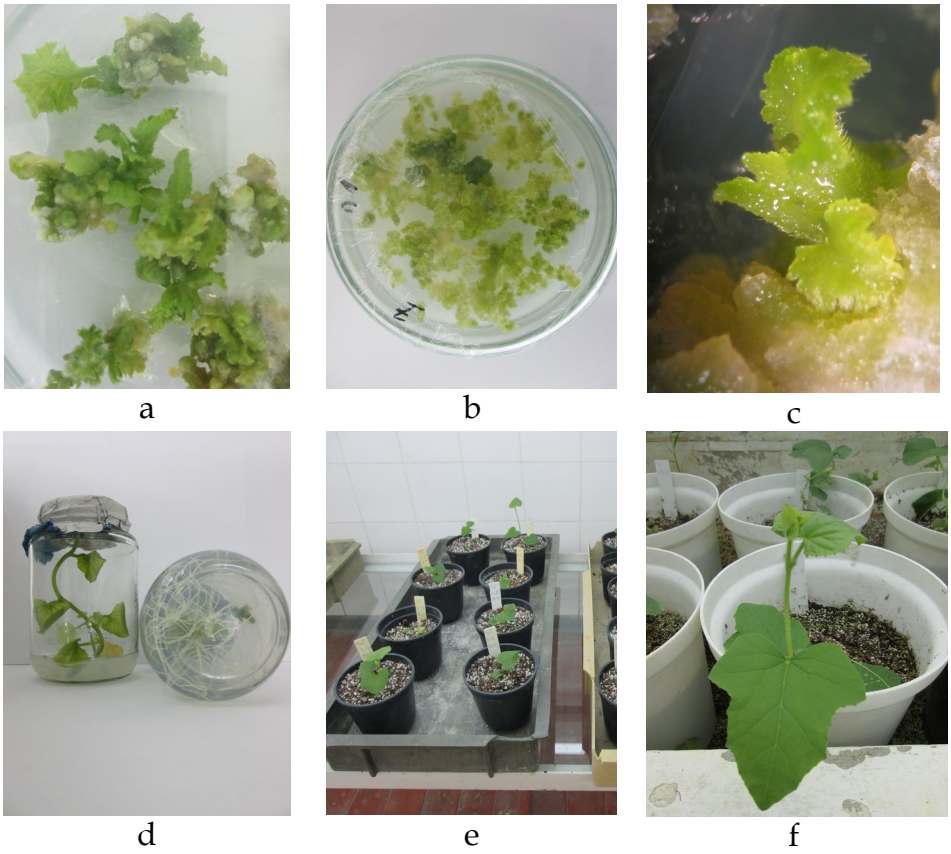

Fig. 3. Regeneration of cotyledons (a), hypocotyls (b), plant-regenerants (c-d) and adapted and acclimatized plants (e-f).

Table 2. Regeneration frequency in cotyledon and hypocotyl explants of two melon lines. Values are means ± standard deviation. Values in columns followed by different letters are significantly different at P≤0.05 based on LSD Test (n=3).

Lines		**11/9**				**AGY**			
Medium		**Regeneration**		**Reg./exp.**		**Regeneration**		**Reg./exp.**	
		%	**±SD**	**No.**	**±SD**	**%**	**±SD**	**No.**	**±SD**
				Cotyledons					
MS0	1	85.0	±21.21^{ab}	1.65	±0.35^{ab}	15.0	±7.07^{ef}	0.35	±0.07^{bc}
	2	60.0	±14.14^{abc}	1.10	±0.14^{bc}	50.0	±7.07^{bc}	0.55	±0.14^{a}
	3	85.0	±21.21^{ab}	1.25	±0.49^{bc}	40.0	±14.14^{cd}	0.40	±0.14^{b}
MS1	1	15.0	±7.07^{c}	0.25	±0.07^{e}	30.0	±7.07^{de}	0.30	±0.07^{bc}
	2	55.0	±7.07^{bc}	0.35	±0.21^{de}	15.0	±7.07^{ef}	0.20	±0.07^{c}
	3	70.0	±14.14^{ab}	0.90	±0.14^{cd}	0.0	±0.00^{f}	0.00	±0.00^{d}
MS2	1	100	±0.00^{a}	1.50	±0.14^{b}	0.0	±0.00^{f}	0.00	±0.00^{d}
	2	55.0	±14.14^{bc}	1.75	±0.35^{a}	0.0	±0.00^{f}	0.00	±0.00^{d}
	3	45.0	±21.21^{bc}	0.85	±0.21^{cd}	0.0	±0.00^{f}	0.00	±0.00^{d}
MS3	1	70.0	±14.14^{b}	1.00	±0.28^{cd}	75.0	±14.14^{a}	0.75	±0.07^{a}
	2	45.0	±7.07^{bc}	0.25	±0.07^{e}	45.0	±7.07^{cd}	0.00	±0.00^{d}
	3	30.0	±14.14^{c}	0.40	±0.28^{de}	65.0	±14.14^{ab}	0.30	±0.07^{bc}

				Hypocotyls			
MS0	1	40.0	±14.14^{ab}	0.35	±0.07^{b}	0.0	0.00
	2	0.0	±0.00^{d}	0.00	±0.00^{e}	0.0	0.00
	3	5.0	±0.00^{d}	0.00	±0.00^{e}	0.0	0.00
MS1	1	0.0	±0.00^{d}	0.00	±0.00^{e}	0.0	0.00
	2	0.0	±0.00^{d}	0.00	±0.00^{e}	0.0	0.00
	3	0.0	±0.00^{d}	0.00	±0.00^{e}	0.0	0.00
MS2	1	50.0	±0.00^{a}	0.75	±0.07^{a}	0.0	0.00
	2	35.0	±7.07^{b}	0.25	±0.07^{c}	0.0	0.00
	3	17.5	±3.54^{c}	0.10	±0.00^{d}	0.0	0.00
MS3	1	0.0	±0.00^{d}	0.00	±0.00^{e}	0.0	0.00
	2	0.0	±0.00^{d}	0.00	±0.00^{e}	0.0	0.00
	3	0.0	±0.00^{d}	0.00	±0.00^{e}	0.0	0.00

Conclusions

To develop an effective and appropriate regeneration system for melon is important to determine both composition of the germination and the regeneration media. The results of this experimental work demonstrated positive influence of pre-treatment with 1 mg L^{-1} BAP on regeneration in cotyledon and hypocotyl explants of melon line 11/9. In line AGY the positive answer was observe only in cotyledons in the highest concentration of BAP. The effect of genotype is difficult to overcome, but combining of suitable explants with culture condition could significantly increase the regeneration frequency. Development of regeneration protocol applicable to a large number of genotypes leads to reducing time and efforts for creation of genetic diversity for breeding purposes. It is also important to conduct more studies of genetic control of the process and requirements of plant growth regulation in different phases of regeneration.

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