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Induction of callogenesis and organogenesis of different melon genotypes

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ABSTRACT

The effect of different concentration of BA and Kinetin on regeneration of cotyledons, hypocotyls and leaves of four melon genotypes was studied. It was established that cotyledons and leaf segments were more effective as explants for bud induction and subsequent plant regeneration than hypocotyls. In the studied melon genotypes, the better regeneration was obtained in medium variants supplemented with growth regulator BA. The best response was observed in true leaves (70.0%) and cotyledons (53.3%) from line 5-1-2 in medium variant containing 1.0 mgL^{-1} BA. On medium variants containing Kinetin the regeneration efficiency was lower. Data from three-way analysis of variance confirmed that regeneration ability is strongly influenced by explant type and interaction of both factors - genotype and explant type. The highest number of plantlets/explant was registered in leaf segments from line K052 (0.9), followed by cotyledons and true leaves from line 5-1-2 (0.7) in medium variant contained 1.0 mgL^{-1} BA.

Key words: *Cucumis melo* L., *in vitro*, regeneration, explant types, growth regulators

Introduction

Melon (*Cucumis melo* L.) is one of the most important species in the *Cucurbitaceae* family. This fact is due to the great diversity of its fruit characters such as colour, shape, size, taste and texture (Paris et al., 2012). By conventional breeding methods, many varieties were developed. However, there is an increasing demand for a new high-yielding varieties combining diseases resistance and fruit quality suitable for growing in different climatic conditions. Tissue culture methods as somaclonal variation and embryogenesis create a new possibility for revealing genetic diversity.

Successful regeneration from melon was reported for the first time by Moreno et al., (1985). Further attempts have been directed towards defining the factors that influence the regeneration ability, including: genotype, explant type, nutrition medium composition, culture condition, etc. (Kintzios & Taravira, 1997; Nunez-Palenius et al., 2008). The role of the genotype as a main factor determines regeneration capacity of melon is proved by many authors (Galperin et al., 2003a; Monforte et al., 2003; Nunez-Palenius et al., 2008). Molina & Nuez, (1995) observed differences in *in vitro* answer even in individual plants from the same genotype.

Plant regeneration in melon has been achieved with different explant types: hypocotyls, cotyledons, leaves, cotyledonary nodes, petioles roots, etc. (Adelberg et al., 1994; Molina & Nuez, 1995; Curuk et al., 2002; Kintzios et al., 2002; Choi et al., 2012; Sebastiani & Ficcadenti, 2013). There are data in the literature on the variation in regeneration answer depending on the position, proximal or distal, and size of the explant (Mendi et al., 2010; Ismail, 2017). Nevertheless, melon regeneration is still not a routine procedure and hormonal requirements in *in vitro* cultivation are poorly studied. Ones of the most commonly used plant growth regulators for induction of regenerative processes are Benzyladenine (BA) and Kinetin, used alone or in combination with Indole-3-acetic acid (IAA) (Guis et al., 1998; Keng & Hoong, 2005; Souza et al., 2006). Investigations of many authors established that some additional factors as light, temperature, humidity and gelling agent also influence the regeneration (Niedz et al., 1989; Curuk et al., 2003). Consequently, for developing an efficient regeneration protocol, not only for melon but also for other *Cucurbitaceae* crops, all the factors impacting the regeneration process, must be studied.

This experimental work is aimed to study *in vitro*

regeneration potential from different explants, which were collected from four melon genotypes on medium supplemented with BA and Kinetin to develop a reliable plant regeneration protocol.

Materials and Methods

Four melon genotypes (BG14, K052, 5-1-2 и SV-3-14) of different varieties were used for developing an efficient plant regeneration protocol. Line 5-1-2 (var. *reticulatus*) characterize with andromonoecious flower types with downy and powdery mildew resistance. Accession BG14 (var. *reticulatus*) is gynoecious line, downy mildew susceptible. Line K052 (var. *common*) is derived from China and produce few fruits, powdery mildew susceptible, andromonoecious. Line SV-3-14 (var. *cantalupensis*) is somaclonal variant originated from male-sterile line 11/9C, monoecious.

Seeds of the four melon genotypes were surface sterilized in 5% calcium hypochlorite solution for 1 hour and rinsed three-times with sterile dH_2O. After that the seeds were sown on basal medium containing macro- and microsalts by Murashige and Skoog (1962), vitamins by Gamborg et al. (1968), 3% sucrose, and 0.7% agar for germination (MS0). Aseptically *in vitro* grown 5-7 days old seedlings were used as a source of explants for cotyledons (0.5 cm^2), hypocotyls (1.0 cm) and true leaves (0.5 cm^2). The explants were cultivated on MS0 basal medium supplemented with different combinations and concentrations of plant growth regulators BA, Kinetin and IAA, for shoot regeneration studies:

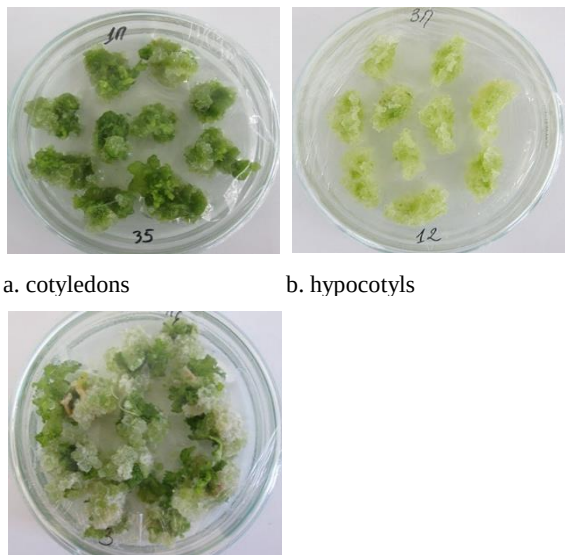
1. 1.0 mgL^{-1} BA + 0.5 mgL^{-1} IAA;
2. 2.0 mgL^{-1} BA + 0.5 mgL^{-1} IAA;
3. 3.0 mgL^{-1} BA + 0.5 mgL^{-1} IAA;
4. 1.0 mgL^{-1} Kinetin + 0.5 mgL^{-1} IAA;
5. 2.0 mgL^{-1} Kinetin + 0.5 mgL^{-1} IAA;
6. 3.0 mgL^{-1} Kinetin + 0.5 mgL^{-1} IAA.

The Petri dishes with explants were incubated in a 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 20 days on the same medium. For further development of obtained shoots, the explants were cultivated on basal medium MS0 containing 0.1 mgL^{-1} BA (Moreno et al., 1985).

The experiment was carried out in three replications with 10 explants 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 was given as mean ± Standard Deviation (SD). Duncan's multiple range test was used to confirm statistical significance of difference among the means. Three-way analysis of variance was performed to determine the differences between genotypes, culture medium variant and explant type as well as the interaction between main factors on organogenesis and regeneration (SPSS 16 software).

Results

The results showed callus induction in all studied medium variants. Depending on the explant type the callus morphology and colour was different. In cotyledons and true leaves the callus was friable and whitish with leaf structures and elongated shoots, while in hypocotyls callus was transparent and watery (Figure 1).



a. cotyledons b. hypocotyls

c. true leaves

Figure 1. *Callus formation in different melon explants*.

The hypocotyl segments reacted with callus formation but the frequency of organogenesis was lower in all studied genotypes and medium variants (0.0% - 33.3%). Both cotyledons (0.0% - 96.7%) and true leaves (0.0% - 100%) gave a higher shoot induction under the same conditions (Figure 2).

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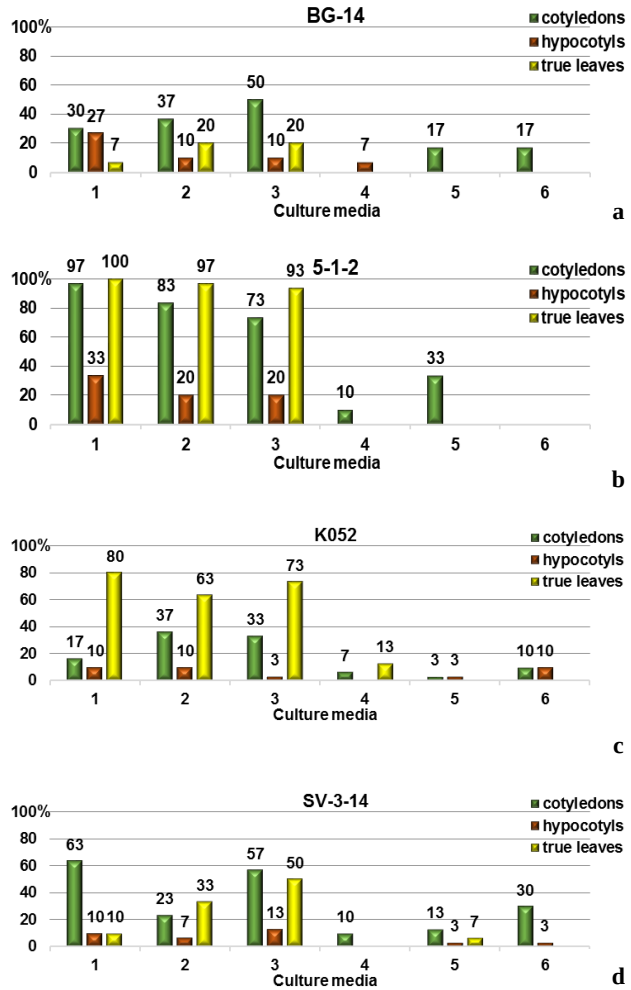

Figure 2. *Organogenic answer in four melon genotypes*.

A better organogenic answer was registered in medium variants containing BA (3.3% - 100%), while in media with Kinetin (0.0% - 33.0%) the organogenesis was lower. In addition, organogenic structures were not established in explants of line 5-1-2 in medium variants supplemented with 3.0 mgL^{-1} Kinetin. At the same time, the explants from line 5-1-2 reacted with the highest percentage of organogenesis in culture media containing BA. Three-way analysis of variance showed that explant type (η=37.25%) influenced mainly organogenic ability in studied melon genotypes (Figure 3a).

The regeneration frequency varied from 0.0% to 70.0% among genotypes, explant type and culture medium composition (Table 1). In the studied melon genotypes, the better regeneration was determined in medium variants supplemented with different concentration of BA. Statistical data showed the highest regeneration rate in true leaves (70.0%) and cotyledons (53.3%) from line 5-1-2 in medium variant containing 1.0 mgL^{-1} BA (Fig. 4). In lines K052 and BG-14 the highest percentage of regenerated cotyledon explants was registered on medium variant containing 2.0 mgL^{-1} BA, while in line SV-3-14 such tendency was not observed. Regeneration in hypocotyl explants in line SV-3-14 in all medium variants containing BA was not established. On medium variants with plant growth regulator Kinetin the regeneration efficiency was lower. Regeneration with higher frequency was registered only in cotyledons from line BG-14 (10.0%) and in true leaves from line SV-3-14 (6.7%) in medium variants containing 3.0 and 2.0 mgL^{-1} Kinetin, respectively. In other genotypes, the plant-regenerants were either not established or the answer was sporadic in all media containing Kinetin.

Data from three-way analysis of variance confirmed that regeneration ability is influenced by explant type (η=24.59%) and interaction of both factors - genotype and explant type (η=20.88%) (Figure 3b).

Table 1. *Regeneration frequency from cotyledon, hypocotyl and leaf explants in four melon genotypes.*

Genotype	Culture medium variants	Cotyledon		Hypocotyl		True leaves	
		%	±SD	%	±SD	%	±SD
BG-14	1	6.7	5.77^{cd}	0.0	0.00^{c}	3.3	5.77^{d}
	2	23.3	20.82^{bc}	3.3	5.77^{b}	6.7	5.77^{d}
	3	16.7	5.77^{bcd}	3.3	5.77^{b}	6.7	11.55^{d}
	4	0.0	0.00^{d}	3.3	5.77^{b}	0.0	0.00^{d}
	5	3.3	5.77^{cd}	0.0	0.00^{c}	0.0	0.00^{d}
	6	10.0	17.32^{cd}	0.0	0.00^{c}	0.0	0.00^{d}
5-1-2	1	53.3	5.77^{a}	3.3	5.77^{b}	70.0	10.00^{a}
	2	13.3	15.28^{bcd}	0.0	0.00^{c}	30.0	30.00^{d}
	3	20.0	20.00^{bc}	10.0	10.00^{a}	36.7	5.77^{c}
	4	3.3	5.77^{cd}	0.0	0.00^{c}	0.0	0.00^{d}
	5	0.0	0.00^{d}	0.0	0.00^{c}	0.0	0.00^{d}
	6	0.0	0.00^{d}	0.0	0.00^{c}	0.0	0.00^{d}
K052	1	3.3	5.77^{cd}	0.0	0.00^{c}	50.0	10.00^{b}
	2	23.3	15.28^{bc}	10.0	0.00^{a}	36.7	5.77^{bc}
	3	3.3	5.77^{cd}	0.0	0.00^{c}	33.3	5.77^{c}
	4	3.3	5.77^{cd}	0.0	0.00^{c}	0.0	0.00^{d}
	5	0.0	0.00^{d}	0.0	0.00^{c}	0.0	0.00^{d}
	6	3.3	3.33^{cd}	0.0	0.00^{c}	0.0	0.00^{d}
SV-3-14	1	20.0	34.64^{bd}	0.0	0.00^{c}	13.3	11.55^{d}
	2	0.0	0.00^{d}	0.0	0.00^{c}	13.3	5.77^{d}
	3	26.7	5.77^{b}	0.0	0.00^{c}	0.0	0.00^{d}
	4	0.0	0.00^{d}	0.0	0.00^{c}	0.0	0.00^{d}
	5	0.0	0.00^{d}	0.0	0.00^{c}	6.7	5.77^{d}
	6	0.0	0.00^{d}	0.0	0.00^{c}	0.0	0.00^{d}

a,b,c…p≤0,05 Duncan's multiple range test

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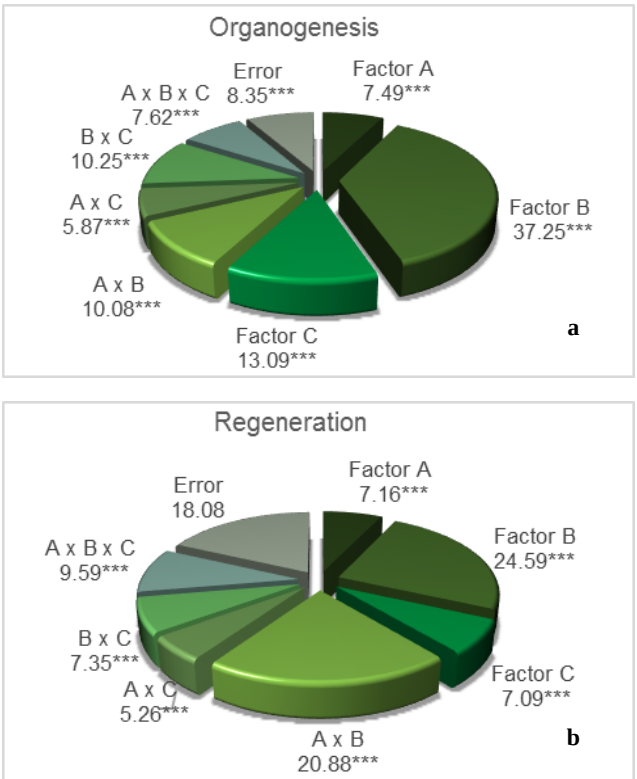

Figure 3. *Three-way analysis of variance and influence of variation factors on the organogenesis and regeneration depend on genotypes (Factor A), explant types (Factor B) and culture media (Factor C).*

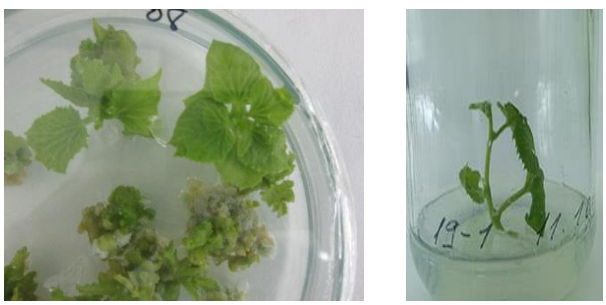

Figure 4. *Induction of in vitro regeneration.*

The same tendency was observed also in number of plantlets/explant (Table 2). The highest number of plantlets/explant was registered in leaf segments from line K052 (0.9) followed by cotyledons and true leaves from line 5-1-2 (0.7) on medium variant supplemented with 1.0 mgL^{-1} BA. In hypocotyls, development of organogenic structures to plantlets was with lower frequency.

Table 2. *Effect on culture medium on number of plants/explants from cotyledons, hypocotyl and true leaves in four melon genotypes.*

Genotype	Culture medium variants	Cotyledon		Hypocotyl		True leaves	
		No	±SD	No	±SD	No	±SD
BG-14	1	0.1	0.15^{bc}	0.0	0.00^{c}	0.1	0.03^{e}
	2	0.4	0.40^{bc}	0.1	0.06^{c}	0.1	0.06^{e}
	3	0.5	0.61^{ab}	0.1	0.06^{c}	0.5	0.87^{b-d}
	4	0.0	0.00^{c}	0.1	0.12^{b}	0.0	0.00^{e}
	5	0.2	0.12^{c}	0.0	0.00^{c}	0.0	0.00^{e}
	6	0.1	0.23^{bc}	0.0	0.00^{c}	0.0	0.00^{e}
5-1-2	1	0.7	0.32^{a}	0.4	0.06^{a}	0.7	0.12^{ab}
	2	0.2	0.32^{bc}	0.0	0.00^{c}	0.1	0.12^{e}
	3	0.2	0.35^{bc}	0.1	0.03^{c}	0.1	0.06^{e}
	4	0.1	0.06^{c}	0.0	0.00^{c}	0.0	0.00^{e}
	5	0.0	0.00^{c}	0.0	0.00^{c}	0.0	0.00^{e}
	6	0.0	0.00^{c}	0.0	0.00^{c}	0.0	0.00^{e}
K052	1	0.1	0.06^{c}	0.0	0.00^{c}	0.9	0.38^{a}
	2	0.1	0.10^{bc}	0.2	0.06^{c}	0.6	0.29^{abc}
	3	0.1	0.04^{c}	0.0	0.00^{c}	0.3	0.06^{cde}
	4	0.1	0.06^{c}	0.0	0.00^{c}	0.0	0.00^{e}
	5	0.0	0.00^{c}	0.0	0.00^{c}	0.0	0.00^{e}
	6	0.1	0.12^{c}	0.0	0.00^{c}	0.0	0.00^{e}
SV-3-14	1	0.2	0.40^{bc}	0.0	0.00^{c}	0.3	0.35^{cde}
	2	0.0	0.00^{c}	0.0	0.00^{c}	0.1	0.06^{de}
	3	0.3	0.15^{c}	0.0	0.00^{c}	0.0	0.00^{e}
	4	0.0	0.00^{c}	0.0	0.00^{c}	0.0	0.00^{e}
	5	0.0	0.00^{c}	0.0	0.00^{c}	0.1	0.06^{de}
	6	0.0	0.00^{c}	0.0	0.00^{c}	0.0	0.00^{e}

a,b,c…p≤0,05 Duncan's multiple range test

Discussion

Melon is still regarded as difficult plant to regenerate and the process is highly related to the genotype (Pech et al., 2007). In the present study a method for regeneration of four melon genotypes using BA, Kinetin and IAA in different concentration was established. Our results showed the highest regeneration frequency in line 5-1-2 followed by line BG-14. These two lines belonging to the taxonomic group *reticulatus*. It is generally considered that *reticulatus* genotypes give better regeneration rates via embryogenesis (Oridate et al., 1992; Gray et al., 1993). On the other hand, Ficcadenti & Rotino (1995) evaluated the morphogenetic response of 11 melon genotypes and established that *C. melo* var. *inodorus* had a uniformly high regeneration rate, whereas *C. melo* var. *reticulatus* varieties showed wide differences in their organogenesis. Genotype is the main factor influencing the efficiency in regeneration process in melon (Galperin et al., 2003a; Sebastiani & Ficcadenti, 2016). Kintzios & Taravira (1997) induced regeneration process only in six among the studied 14 melon genotypes and concluded that regeneration

ability was genotype-dependent which was confirmed in the present study. Galperin et al., (2003b), screened 30 different commercial melon cultivars for shoot *de novo* regeneration, but only one inbred line BU-21 had high regenerative capacity and formed several shoots per explant. The author found that a single dominant gene Org-3 controls multiple shoot inductions in this line. Nunez-Palenius et al., (2008) also established that melon *in vitro* response is under genetic control.

The positive effect of plant growth regulator BA on melon regeneration was demonstrated in the current study. The comparison of varieties and media confirmed that BA combined with IAA was able to induce regeneration in the studied melon lines. BA either alone or in combination with auxin has been previously reported to be optimal for *in vitro* regeneration in melon (Abrie & Staden, 2001; Souza et al., 2006; Valdez et al., 2009).

The influence of Kinetin on regeneration process was observed in other species of *Cucurbitaceae* family (Grozeva & Velkov, 2014; Firoz Alam et al., 2015; Abu-Romman et al., 2015) but in melon, it has contradictory effects. Moreno et al. (1985) reported that Kinetin, in combination with IAA, stimulates regeneration potential in melon, while Neidz et al. (1989) and Abrie & Staden (2001) established that BA was more effective than Kinetin in the conditions of their experiments. Our results showed the highest percentage of regeneration explants in medium supplemented with 1.0 mgL^{-1} BA and 0.5 mgL^{-1} IAA. Ficcadeni & Rotino (1995) reported that BA stimulates the regeneration process but in combination with Abscisic acid (ABA) significantly increases the number of regenerants per explant. According to some authors, the addition of auxins in culture media stimulates callogenesis, but does not influence the regeneration process (Keng & Hoong, 2005; Ren et al., 2013). On the other hand, Mendi et al., (2010) obtained more regenerants per explant on medium supplemented with combination of 0.5 mgL^{-1} BA and 0.5 mgL^{-1} IAA (88%), compared to the use of 1.0 mg L^{-1} BA (75%) alone. Kiss-Bába et al., (2010) underlined the positive effect of IAA adding to BA on shoot induction and regeneration.

Appropriate choice of the explant significantly influences the morphogenic ability in many vegetable crops (Kiss-Bába et al., 2010; Galperin et al., 2003). Some authors established that the cotyledons and true leaves are the most responsive explants compared to hypocotyls. In melon, younger, smaller leaves and very young cotyledons were found to be most responsive (Souza et al., 2006; Pech et al., 2007). Similar results were obtained in this study. The cotyledons and true leaves gave more regenerants than hypocotyls. Differences observed in regeneration capacity of the explant types probably due to the influence of endogenous growth regulators and combination of these, added in a culture medium, but also of the position of the explant in the plant (Torelli et al., 2004).

Conclusion

The present study reports an optimized protocol for organogenesis through callus for four melon genotypes. Statistical significant differences in regeneration frequency among the melon genotypes depending on genotype, explant type and medium variant were registered. It can be concluded that in melon regeneration, the genotype is the main factor determined regeneration answer, but explant type and medium variant are also important for this process. The higher frequency of shoot regeneration was achieved from cotyledons and leaf segments. The line 5-1-2 was genotype who gives better regeneration answer. In general, the regenerated plants from all tested cultivars with higher frequency was established in medium variants supplemented with different concentration of BA compared to Kinetin. The best regeneration results were achieved on culture medium 1.0 mgL^{-1} BA + 0.5 mgL^{-1} IAA for line 5-1-2, and 2.0 mgL^{-1} BA + 0.5 mgL^{-1} IAA for lines K052 and BG-14.

The plant regeneration protocol developed in the present study could be used for lines BG14, K052, 5-1-2 и SV-3-14, but further studies are needed to identify the optimal regeneration protocol for other genotypes.

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