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In Vitro Regeneration of Iranian Melon (*Cucumis Melo* L. 'Samsoori') Using Antibiotic and Benzyl adenine Micropropagation of *Cucumis Melo* L. 'Samsoori'

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Abstract

Cucumis melo L. is one of the most economically important horticultural crops worldwide. However, low plant regeneration frequency of this plant during genetic transformation is the major hurdle for applying biotechnological approaches. Hence, this study aimed to evaluate the effect of 6-benzyladenine (BA), cefotaxime (CTX), kanamycin (KAN), and indole-3-acetic acid (IAA) on the regeneration of cotyledonary petioles generated from 6-day-old *in vitro* grown seedlings. Results showed that application of 1.5 mgl^{-1} BA plus 250 mgl^{-1} CTX and 1 mgl^{-1} BA with 1000 mgl^{-1} CTX formed the most efficient media for plant regeneration. The highest callus production was recorded on medium containing 1 mgl^{-1} BA with 250 mgl^{-1} CTX and 1.5 mgl^{-1} BA with 750 mgl^{-1} CTX. Medium containing 500 mgl^{-1} CTX plus 0.1 mgl^{-1} IAA efficiently induced both root \ and leaf formation. All regenerated plants were died by adding 100 mgl^{-1} kanamycin therefore this level considered as threshold level for kanamycin application.. Overall, the results indicated that presence of BA plays an essential role for melon regeneration and cefotaxime can be considered as an auxiliary agent.

Keywords: cefotaxime, *Cucumis melo*, kanamycin, micropropagation, organogenesis.

Abbreviations: **ABA**, Abscisic acid; **BA**, 6-benzyladenine; **BAP**, 6-benzylaminopurine; **CTX**, Cefotaxime; **cv**, Cultivar; **d**, Day(s); **GA_3**, Gibberellic acid; **h**, Hour(s); **IAA**, Indol-3-acetic acid ; **IBA**, Indole-3-butyric acid; **KAN**, kanamycin; **min**, Minute(s); **MS**, Murashige and Skoog medium (1962); **NAA**, α-naphthaleneacetic acid; Polymerase chain reaction; **PGR**, Plant growth regulator(s).

Introduction

C*ucumis melo* L. is one of the most economically important species within the Cucurbitaceae family This family includes a large number of cultivated varieties and wild genotypes commonly spread in tropical, subtropical and warm temperate (hot summer) regions of the globe. The melon most likely originated from Central Asia, particularly from Khorasan region of Iran (Paris and Amar, 2012).

During 2012, the yield performance of cantaloupe and other melons in Iran has been estimated to around 17682.9 $Kg.ha^{-1}$ with a total production quantity of 1,450,000 tonnes. Asia has dominated for the world production of cantaloupe and other melons. Among the leading countries in Asia, the

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highest production (17,568,700 tonnes) is belonging to the China followed by Turkey and Iran (FAO, 2012). During plant transformation process, establishment of an efficient *in vitro* plant regeneration system plays a pivotal role. In melon regeneration, morphological abnormalities, such as the presence of meristematic protuberances that fails to develop into normal shoots or generation of plantlets without apical dominance are prevalent hurdles. (Stipp *et al*., 2001). To develop a simple and routine procedures regeneration capacity and transformation efficiency of melons are the main challenges which are highly dependent on explant sources (Zhang *et al*., 2014). Moreover, the effects of growth condition and plant growth regulators (PGRs) during melon regeneration are not fully discovered yet. Recent studies indicate that the regeneration capacity and transformation efficiency of melon below the 12.5% (Nuňez-Palenius *et al*., 2006) could be due to the (i) premature cell vocalization and disorganization of most of the meristematic structures and (ii) disruption of GUS-positive meristematic areas after 14 days (Chovelon *et al*., 2011). In a recent study, cotyledonary petiole explants of *C. melo* 'Khatooni' cultured on 0.1 mgl^{-1} BA plus 5 mgl^{-1} 2, 4-dichlorophenoxyacetic acid (2, 4-D) had the highest efficiency for somatic embryogenesis (Naderi *et al*., 2011). Positive and negative effects of antibiotics such as cefotaxime (CTX) on plant growth, have been revealed in several studies and it has been shown that the appropriate concentration of this antibiotic depends on the plant species, type of explant, and culture system (Tang *et al*., 2000). However, a successful regeneration protocol, which can also be applied for melon transformation, is an urgent need. In this regard, this study aimed to (i) find an effective regeneration procedure applicable for cotyledonary petiole explants of Iranian melon, *Cucumis melo* 'Samsoori', (ii) find an optimal concentration of cefotaxime in order to evaluate its impact on the regeneration of melon and (iii) determine an appropriate threshold of kanamycin (KAN) for evaluation of melon tolerance to kanamycin application.

Materials and methods

Plant material and preparation

Mature seeds of *Cucumis melo* 'Samsoori' were used as the explant sources for organogenesis induction. After removing seed coats, the seeds were disinfected by 70% ethanol for 2 min, followed by 1.5% sodium hypochlorite solution plus 2 drops of Tween-20 per 100 ml solution for 20 min. Finally, the seeds were rinsed four times in sterile distilled water and transferred to ½ MS medium (Murashige and Skoog, 1962).

The proximal cotyledon section with its 2-3 mm-long hypocotyl stub of 6-d-old *in vitro* grown seedlings (Fig. 1A) was excised as an explant, and the apical bud of the seedling was removed under Stereomicroscope ('de-budding'). Explants derived from cotyledonary petioles (5-7 mm) were incubated in abaxial side down on MS medium supplemented with different concentrations of BA (0, 1, and 1.5 mgl^{-1}), and CTX (0, 250, 500, 750, and 1000 mgl^{-1}). Regenerated shoots grown on medium containing 250 mgl^{-1} CTX + 1 mgl^{-1} BA were sub-cultured on MS medium supplemented with different concentrations of CTX (0, 250, and 500 mgl^{-1}), KAN (0, 50, and 100 mgl^{-1}), and 0.1 mgl^{-1} IAA for root induction.

The medium pH level was adjusted to 5.8 with 1 N KOH or HCL prior to autoclaving at 121°C for 20 min. Cultures were incubated in the growth chamber at 25±1°C in a normal 16/8 h photoperiod provided by 40-50 $\mu mol.m^{-2}.s^{-1}$ cool-white fluorescent lamps. Each combination of concentrations was replicated 8 times in petri dishes (100 ×15 mm), with 4 segments in each replicate. Explants were sub-cultured in the same medium every three weeks after data collection. The percentage of shoots and buds generated from the explants, number of buds and shoots per explant, explant expansion and

callus growth were measured at three-week intervals.

Statistical analysis

Each treatment replicated three times. The data were analyzed for mean and standard error (SE). A factorial experiment in a completely randomized design was applied to analyze the data. Analysis of variance (ANOVA) using SAS (version 9.1) was applied to test the data and the difference among the means was compared using LSD test at $P < 0.05$.

Results

Effects of BA and CTX on shoot regeneration and callus induction

Selection of appropriate antibiotics with optimum application level play a pivotal role in reducing of negative impact on regeneration processes (Silva and Fukai, 2001, Wiebke *et al*., 2006, Choi *et al*., 2012). In this regard, the present study was designed to determine the optimum dosage of BA and CTX for regeneration of *Cucumis melo* 'Samsoori'. CTX induced the bud initiation, shoot regeneration, callus formation, as well as root and leaf formation (Tables 3 and 6 and Figures 1, 2, 3, 4, and 5). On MS medium supplemented with BA, the percentage of explants containing emerged buds and shoots and the number of shoots and buds per explants markedly increased (6.6) at the concentration of 1 mgl^{-1} BA (Table 2). Bud initiation and the formation of white friable callus were started after about two weeks (Fig. 6B). Among all applied concentrations, the highest number of shoot regeneration per explants and percentage of explants forming shoots were observed on MS medium containing 250 and 1000 mgl^{-1} CTX, respectively (Table 3). As shown in Figure 1, the highest percentage of explants forming shoots was observed in medium containing 1 mgl^{-1} BA plus 1000 mgl^{-1} CTX. The highest number of shoots per explants was observed at 1.5 mgl^{-1} BA with 250 mgl^{-1} CTX, but without significant difference with the medium containing 1 mgl^{-1} BA plus 1000 mgl^{-1} (Figure 2). The results significantly showed that the medium containing BA and CTX had higher regeneration efficiency compared to MS medium without BA (Figures, 1, 2, 3 and 6). Despite of the high frequency of shoot generation per explant at 1.5 mgl^{-1} BA plus 250 mgl^{-1} CTX (Figure 2), the combination of 1 mgl^{-1} BA with 1000 mgl^{-1} CTX showed a superior effect on improving the percentage of explants forming shoots and number of shoot per explant (Figures 1 and 2). Regarding the callus formation, the highest callus number was observed on medium containing 1 mgl^{-1} BA plus 250 mgl^{-1} CTX and 1.5 mgl^{-1} BA plus 750 mgl^{-1} CTX (Figure 3).

Table 1. Interaction effects of PGRs and antibiotic associated with regeneration ability based on two-way analysis of variance

SOV	DF	No. of explants forming roots	No. of explants forming leaves	No. root per glass container	No. leaf per glass container	Plantlet length	No. plantlet shoots per glass container
CTX	2	972.22^{*}	10.22^{*}	0.23^{ns}	0.35^{*}	0.04^{*}	0.24^{**}
KAN	1	13888.89^{**}	73.36^{**}	3.31^{**}	2.49^{**}	0.26^{**}	1.16^{**}
CTX×KAN	2	972.22^{*}	4.85^{ns}	0.23^{ns}	0.14^{ns}	0.01^{ns}	0.18^{*}
Error	12						

contd.

SOV	DF	Explant expansion	No. explants forming buds	No. explants forming shoots	No. of buds per explant	Callus fresh weight	Callus growth
BA	2	14.26^{**}	42158.85^{**}	277.19^{**}	43.15^{**}	6.24^{**}	3.33^{**}
CTX	4	1.92^{**}	248.67^{ns}	3.22^{*}	1.11^{**}	0.33^{**}	0.06^{**}
BA×CTX	8	0.28^{**}	151.13^{ns}	2.11^{ns}	1.02^{**}	0.22^{**}	0.04^{**}
Error	29						

DF: degree of freedom; SOV: source of variation; * and ** indicate significant differences at $P \leq 0.05$ and $P \leq 0.01$; ns: not significant.

Table 2. Effects of various BA concentrations on shoot induction and callus formation of *C. melo*'s explant after 3 weeks

Treatment BA (mgl^{-1})	Explant expansion	% of explants forming buds	No. of buds per explant	% of explants forming shoots	No. of shoots per explant	Callus fresh weight (g)	Callus growth
0	1.2±0.1b	0b	0b	0b	0c	0b	0b
1	2.8±0.7a	94.1 a	14.1 ±2.6a	73.7 a	6.6±1.3a	4.12±2.1a	1.9a
1.5	3.2±0.1a	92.4 a	13.3±2.1a	66.9 a	4.1±1.1b	2.91±0.1a	2.1a

Explant expansion and Callus growth, 0: without callus, 1: Slight regenerative, 2: Moderate, 3: Profuse. Values within column sharing same letters are not statistically different at 5% probability level.

Table 3. Effects of various CTX concentrations on shoot induction and callus formation of *C. melo*'s explant after 3 weeks

Treatment CTX (mgl^{-1})	Explant expansion	% of explants forming buds	No. of buds per explant	% of explants forming shoots	No. of shoots per explant	Callus fresh weight (g)	Callus growth
0	1.7±1.1d	67.9 a	6.6±2.1b	31.5b	1.2±0.5c	2.23±1.9b	1.2b
250	2.4±0.1b	65.2 a	13.1±1.8a	49.5a	7.6±1.2a	3.45±1.5a	2.9a
500	2.4±0.7b	51.8 b	6.1±1.2b	53.0a	3.6±1.1b	1.69±1.1bc	1.2b
750	2.9±0.8a	60.9 ab	9.8±0.8a	51.1a	3.9±1.3b	1.69±1.1bc	1.2b
1000	2.1±0.1c	65.1 a	9.8±0.8a	54.2a	3.7±1.2b	1.33±1.1c	1.2b

Explant expansion and Callus growth, 0: without callus, 1: Slight regenerative, 2: Moderate, 3: Profuse. Values within column sharing the same letters are not statistically different at 5% probability level.

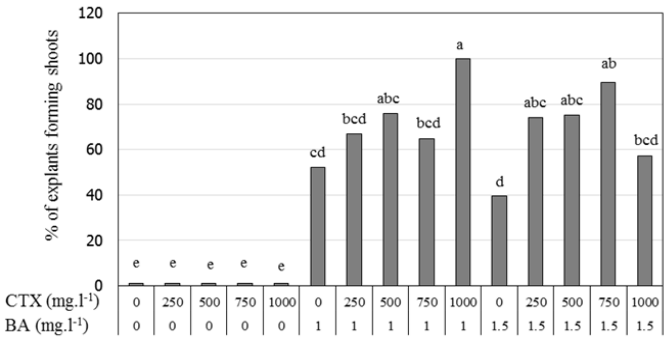

Fig. 1. Interactive effects of BA and CTX associated with the percentage of explants forming shoots in *C. melo* 'Samsoori' after 3 weeks. Values sharing the same letters are not statistically different at 5% probability level.

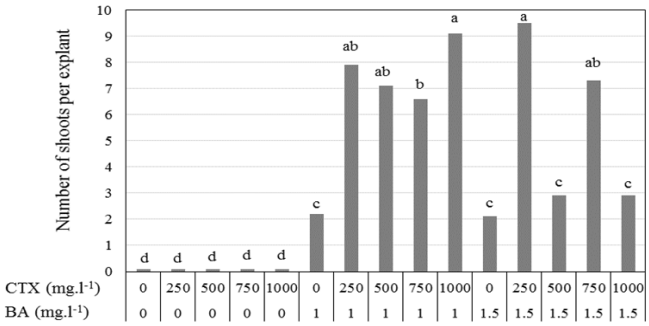

Fig. 2. Interactive effects of BA and CTX on the number of shoots per explants in *C. melo* 'Samsoori' after 3 weeks. Values sharing the same letters are not statistically different at 5% probability level.

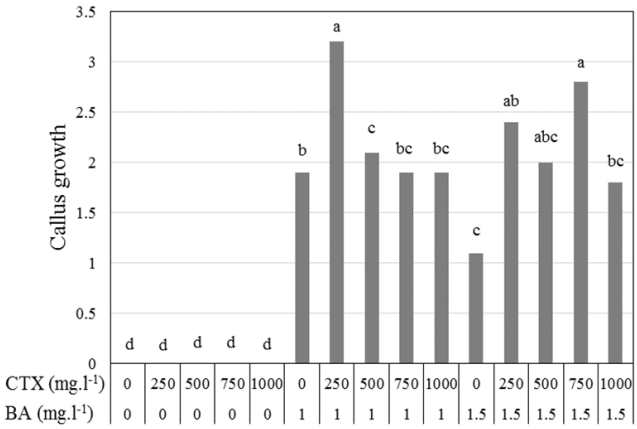

Fig.3. Interaction effects of BA and CTX associated with the callus growth of *C. melo* 'Samsoori' after 3 weeks. 0: without callus, 1: Slight regenerative, 2: Moderate, 3: Profuse. Values sharing the same letters are not statistically different at 5% probability level.

Effects of IAA, CTX, and KAN on regenerated shoots

Preliminarily experiments of this species displayed efficient formation on MS medium either containing 0.1 mgl^{-1} IAA or hormone-free medium. Therefore, 0.1 mgl^{-1} IAA was applied in this study for root induction. , However, application of CTX did not result in any phytotoxic effect on regeneration system (Figures 6B and 6C) the frequency of regeneration rate was improved and showed a synergistic effect with BA (Figures 1, 2 and 3) and IAA (Figures 4 and 5). Although the concentration of 500 mgl^{-1} CTX with 0.1 mgl^{-1} IAA considerably induced the root induction (Table 4 and Figure 6C), 250 CTX mgl^{-1} plus 0.1 mgl^{-1} IAA significantly promoted both shoot, and leaf formation (Figures 4 and 5).

To identify the optimum concentration of kanamycin required for emergence of the explants resistance, three concentrations of kanamycin were tested. Results indicated that the explants growth were completely suppressed with the application of 100 mgl^{-1} kanamycin (Table 4).

Table 4. Interaction effects of various concentrations of KAN and CTX on root and shoot induction of *C. melo* 'Samsoori' cotyledonary petiole explants after 3 weeks.

Treatment			% of explants forming roots	No. plantlet shoots per glass container
IAA (mgl^{-1})	CTX (mgl^{-1})	KAN (mgl^{-1})		
0.1	0	0	33.3b	1.2b
0.1	250	0	50b	3.1a
0.1	500	0	83.3a	1.1b
0.1	0	50	0c	0.2c
0.1	250	50	0c	0.6c
0.1	500	50	0c	0.6c
0.1	0	100	0c	0d
0.1	250	100	0c	0d
0.1	500	100	0c	0d

Values within column sharing the same letters are not statistically different at 5% probability level.

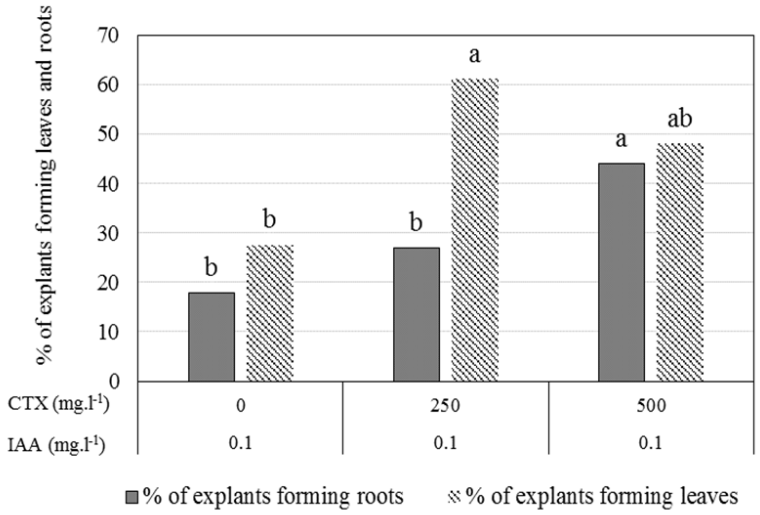

Fig. 4. Interactive effects of various CTX concentrations combined with IAA on the percentage of leaf and root formation in *C. melo* 'Samsoori' cotyledonary petiole explants after three weeks.

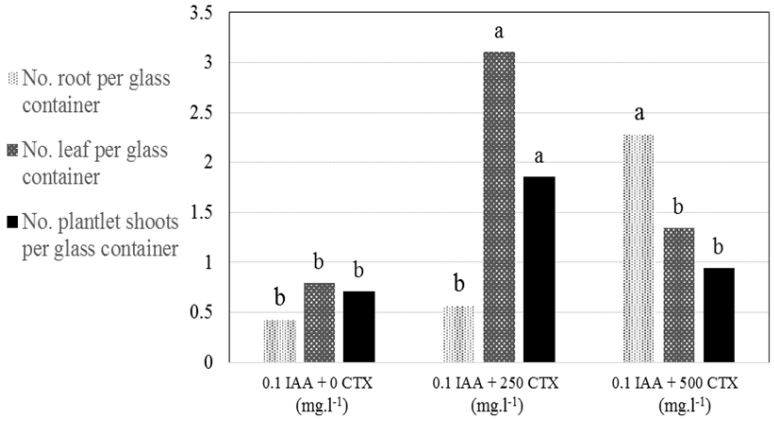

Fig.5. Interactive effects of various CTX concentrations combined with IAA represent the number of root, leaf, and shoot formation in *C. melo* 'Samsoori' cotyledonary petiol explants per glass container after 3 weeks.

Discussion

Effects of BA and CTX on shoot regeneration and callus induction

Several studies demonstrated that antibiotics like CTX represents both positive and negative effects on *in vitro* plants regeneration either directly or indirectly. These effects are mainly dose-dependent and are various according to plant species, type of the explant, and medium composition. Study on plant regeneration of wheat mature embryos showed that the application of timentin and CTX had no adverse effects compared with carbenicillin and clavamox, while the highest growth suppression of *Agrobacterium* and callus induction was obtained by 250 mgl^{-1} CTX application (Han *et al*., 2007). Moreover, the positive effect of CTX on promotion of somatic embryogenesis of *Dianthus* cultivars at 100-500 mgl^{-1} (Nakano and Mii, 1993) and *Triticum aestivum* at 60-100 mgl^{-1} (Mathias and Boyd, 1986) have also been reported. More vigorous regeneration of hypocotyl in comparison with cotyledon could be due to the presence of young and undifferentiated cells in its proximal zone

(Curuk *et al*., 2002), different endogenous phytohormone level (Liu *et al*., 2013), or the meristematic activity of plant tissue, which might be affected by PGRs such as cytokinin (Koné *et al*., 2013). Besides, meristematic cells called meristemoids have a greater potential of bud induction. The superiority of cotyledon explant to the hypocotyl and zygotic embryo explant for embryogenesis of Tunisian *Cucumis melo* L. cultivars Beji and Maazoun has been corroborated. In melon and cucumber, callus tissues originated from hypocotyl explants and attached to their cotyledon fragment, were highly regenerative. On MS medium containing 4.4 µmol BA, regenerated shoots obtained from hypocotyl segment were almost 100% diploid, while developed shoots from cotyledon displayed 40% to 70% polyploidy (often tetraploid) affected by somaclonal variation (Rhimi *et al*., 2006; Curuk *et al*., 2003). Consistent with our observations, the superiority of cotyledonary petiole explants over other explant types in *Cucumis melo* cv. Khatooni from Cucurbitaceae have been also reported (Naderi *et al*., 2011). ,.

In this study, regeneration media supplemented with 1 mgl^{-1} BA plus 1000 mgl^{-1} CTX and also 1.5 mgl^{-1} BA plus 250 mgl^{-1} CTX showed high efficiency in melon regeneration (Figures 1 and 2). The different responses to BA reported in melon studies can be possibly due to the various level of endogenous hormone among different cultivars. Regeneration rates may also be affected by plant genotype and varieties, explant age, and gelling agents (Soza *et al*., 2006).

The quality and quantity of callus formation in *Cucumis* genus have been strongly intertwined with genotype, explant source, and the PGRs (Abu-Romman *et al*., 2013). An experiment on cucumber showed that NAA and IAA, had the best efficacy on callus formation and production of yellow and friable callus tissues. However, brown and compact calluses formation reduced callus growth index and quality in medium supplemented with BA (Abu-Romman *et al*., 2013). In this experiment, the higher rate of white and friable callus formation was observed on medium containing 1 mgl^{-1} BA plus 250 mgl^{-1} CTX (Figure 3).

Effects of IAA, CTX, and KAN on regenerated shoots

Regarding the positive impact of CTX on plant regeneration, the outcomes were consistent with those obtained by Yepes and Aldwinckle (1994) where the regeneration and shoot development in apple tissues were encouraged by applying 250 mgl^{-1} CTX. Besides having a positive aspect of *Agrobacterium* elimination by applying 250 mgl^{-1} CTX as reported by Han and coworkers (2017), the results of the present paper verified beneficial influence of CTX on regeneration processes of *C. melo* 'Samsoori'. Hypothetically the positive impact of antibiotics is accounted for their plant hormone-like effects on plant tissues, which can also be affected by the PGRs in media and influence the regeneration and callus growth (Mathias *et al*., 1986). Additionally, the positive interaction effects of CTX and BA could be due to their synergistic effect. Although root induction was easily facilitated by 0.1 mgl^{-1} IAA, CTX exposure remarkably improved root induction along with shoot, leaf, and callus formation and shoot elongation. The significant positive synergistic effect of BA and CTX were observed on regeneration of *C. melo* 'Samsoori' (Tables 1 and 4 and Figures 1, 2, 3, 4, and 5). Similar results have been reported by Ren *et al*. (2012), by using the MS basal medium supplemented with 1 mgl^{-1} BA, 0.26 mgl^{-1} ABA, and 0.8 mgl^{-1} IAA for shoot regeneration from cotyledonary petiole explants of cantaloupe and honeydew melons. Although surfing in the literatures shows that MS medium supplemented with IAA 1 mgl^{-1} efficiently induced melon root induction, the best root induction in this study occurred with IAA

0.1 mgl^{-1} on MS medium (data not shown). This could be due to the different responses of melon cultivars to PGRs (Venkateshwarlu, 2012).

The 'Samsoori' melon explant growth was entirely repressed by 100 mgl^{-1} KAN supplement (Table 4). The present findings were consistent with those reported by Tabei *et al*. (1998) described that 100 mgl^{-1} KAN was efficiently eliminate the non-transgenic shoots of cucumber. However, 25 mgl^{-1} KAN had a positive influence on shoot regeneration, whereas none of the tested concentrations had a positive response in the present experiments. In a different study, early development of non-transformed shoots of cucumber was observed on 100 mgl^{-1} KAN, while 200 mgl^{-1} KAN was efficient for transgenic shoot selection (Rajagopalan and Perl-Treves, 2005). Studies pertinent to the melon transformation indicate that the efficiency of KAN for transformant selection is species dependent and may vary within a wide range from 25 to 300 mgl^{-1} (Akasaka-Kennedy, 2004; Guis *et al*., 1998). Therefore, according to these results it can be inferred that plant sensitivity to antibiotics is species-specific and 100 mgl^{-1} KAN can suppress regeneration of *Cucumis melo* (L.) 'Samsoori'. Plantlets with a well-developed root system (Figure 6D and E) successfully adapted (100% survival rate) to transplanting them into plastic glasses containing peat moss and perlite (1:1) in a greenhouse under normal condition and ambient temperature of 23±2°C with 70% relative humidity.

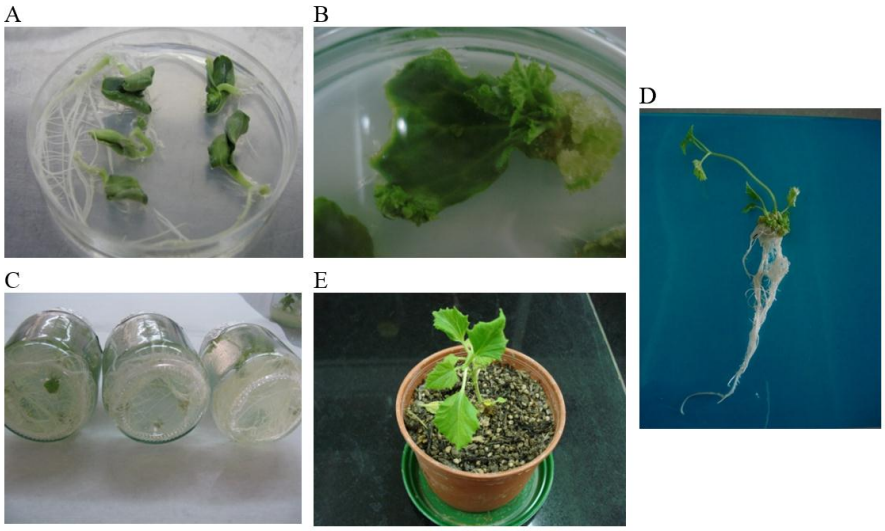

Fig. 6. Plant regeneration from cotyledonary petioles of *Cucumis melo* var. Samsoori: (A) 6-d-old seedling germinated on ½ MS; (B) Regenerated explants cultured on MS medium containing 1 mgl^{-1} BA plus 500 mgl^{-1} CTX after 4 weeks; (C) Rooting of regenerated shoots on medium containing 0.1 mgl^{-1} IAA plus 500 mg l^{-1} CTX after 18 d; (D) Whole plantlet before transferring to greenhouse; (E) Hardened plant after transferring to room conditions

In conclusion, this study demonstrated the optimal dosage of BA, CTX, and KAN for commercial regeneration of transgenic and non-transgenic Iranian melon. Application of BA and CTX positively affected the regeneration of melon, which is also applicable for *Agrobacterium* elimination. In addition to the positive effect of CTX on shoot induction and its combination with IAA plus BA had notable synergistic effects on bud and root induction, shoot elongation, leaf development and callus growth which was possibly due to a hormone-like effect. Moreover, regeneration was completely suppressed on medium containing 100 mgl^{-1} KAN (Table 4), indicating a threshold for kanamycin in killing of the non-transgenic

tissues as an appropriate dosage for selection of transformed plantlets. Overall, our results suggested an optimal level of BA and antibiotics, which are applicable for regeneration and transformation of *Cucumis melo* (L.) 'Samsoori'.

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