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Genetic transformation of Persian melon (*Cucumis melo* L.) via *Agrobacterium*

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ABSTRACT INFO

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ABSTRACT

A reliable *Agrobacterium*-mediated transformation and regeneration protocol was developed for commercially important endemic Persian melon cultigens (*Cucumis melo* L.) comprising 'Eyvanaki', 'Samsoori', and 'Khatooni'. The effect of selective Murashige and Skoog (MS) medium containing various concentrations of 6-benzyl adenine (BA) (0, 0.5, 1, and 1.5 mg l^{-1}) and 1 mg l^{-1} Gibberellic acid (GA_3) on regeneration of cotyledon, hypocotyl, and cotyledonary petioles derived from 6-day-old *in vitro* grown seedlings of the three Persian melons were investigated. For transformation, the sensitivity to kanamycin (Km) concentrations (0, 50, 75, 100, 125 mg l^{-1}), the effect of three *A. tumefaciens* strains (GV3103, LBA4404, and AGL0), inoculation time (0.5, 1, 5, and 30 min), and co-cultivation time (24, 48, and 72 h) on direct shoot regeneration of cotyledonary petiole of 'Samsoori' were investigated. Shoot regeneration from cotyledonary petiole explants received the highest attention. Cotyledonary petiole segments of 'Samsoori' and 'Khatooni' treated respectively with 1.0 mg l^{-1} and 1.5 mg l^{-1} BA exhibited the highest potential for shoot multiplication; while the regeneration rate of 'Eyvanaki' was drastically lower. Putative transgenic 'Samsoori' plantlets selected in 100 mg l^{-1} Km were subcultured on elongation MS medium composed of 100 mg l^{-1} Km, 0.1 mg l^{-1} BA, 1 mg l^{-1} GA_3 plus 400 mg l^{-1} CTX, and then successfully rooted on growth regulator-free MS medium for two weeks. Using histochemical GUS assay along with genomic PCR screening for *GusA* and *VirG* genes, the efficiency of transformation was estimated to be 10% for AGL0 and 6% in LBA4404.

Key words: AGL0, *Cucumis melo*, Multiple buds, Organogenesis, Reporter genes, Transgenic plant.

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ABBREVIATIONS

BA (6-benzyl adenine), CTX (Cefotaxime), GA_3 (Gibberellic acid), GUS (Glucuronidase), Km (Kanamycin), MS (Murashige and Skoog), NPTII (Neomycin phosphor transferase), PCR (Polymerase chain reaction).

INTRODUCTION

Among Cucurbitaceae, *Cucumis melo* L. is one of the most important cultivated species. In addition to the considerable amount of vitamins and minerals, melon fruits are a valuable source of bioactive compounds such as elatrin, stigmastrol, carotenoids as well as cucurbitacin B as effective antioxidant and anticancer agents (Robinson and Decker-Walters, 1999; Mallek-Ayadi *et al.,* 2022). Melon seeds are also known as a good source of protein, oil, and fiber (Silva *et al.,* 2020). Interspecific hybridization or conventional breeding methods are useful for transferring organoleptic traits along with obtaining optimal yield and resistance. Yet, successful hybridization and germplasm improvement take a long time and great effort, and are restricted in melons due to strong sexual incompatibility barriers at the interspecific and intergenetic levels (Robinson and Decker-Walters, 1999; Nuñez-Palenius *et al.,* 2008; Kesh *et al.,* 2021) and is limited to transferring a few numbers of genes (Nuñez-Palenius *et al.,* 2008; Komala *et al.,* 2022). The high-quality attributes of commercial melons such as non-bitterness, sweetness or high-sucrose fruits, and low-acidic fruits are manifested mostly by three genes including bif/bif, suc/suc, and so/so, respectively (Burger *et al.,* 2003; Choudhary *et al.,* 2020). Since negative attributes such as bitterness, high acidity, and low-sucrose traits are controlled by dominant genes in wild melons (Dogimont, 2011), any interspecific hybridization with wild melons can lower the quality of fruits. Nowadays, biotechnological techniques have been used as one of the most promising emerging tools for overcoming these barriers and obtaining cucurbit crops with superior quality traits, yield, and resistance (Navratilova *et al.,* 2011; Komala *et al.,* 2022; Feng *et al*., 2023; Adiguzel *et al.,* 2023).

Recently, the amplified fragment length polymorphism (AFLP) marker uncovered the genetic diversity of previously well-known Persian melon cultivars such as 'Khatooni', 'Samsoori', and 'Eyvanaki', demonstrating a wide diversity of Persian melon cultigens (Danesh *et al.,* 2015). To date, some successful protocols for plant regeneration and genetic transformation of *C. melo*. have been reported (Akasaka-Kennedy *et al.,* 2004; Garcia-Almodovar *et al.,* 2017; Xiao *et al.,* 2023; Shirazi Parsa *et al.,* 2023; Wan *et al*., 2023) however, most of them are highly genotype-dependent and there appears to be few reports on stable transformation of Iranian local cultivars. In this study, the transformation and organogenesis of 'Eyvanaki', 'Samsoori', and 'Khatooni' were investigated. This research aimed to determine the Iranian melon with the highest regeneration capacity and the optimal conditions for plant regeneration, the best explant type for organogenesis, and the optimum inoculation and co-cultivation conditions for T-DNA transfer.

MATERIALS AND METHODS

Plant material

As indicated in Table 1, three Persian endemic melon cultigens (*Cucumis melo* L.) comprising 'Eyvanaki', 'Samsoori', and 'Khatooni' were selected in this study. Mature seeds were provided by the Seed and Plant Improvement Institute (SPII) and used as a source of explants. De-coated seeds were surface-sterilized by shaking in 70% ethanol for 2 min, followed by 1.5% sodium hypochlorite solution plus two drops (0.1% v/v) of Tween-20 per 100 ml solution for 20 min. The seeds were finally rinsed four times in sterile distilled water and cultured on ½ MS (Murashige and Skoog, 1962) medium supplemented with 30 g l^{-1} sucrose and

Table 1. List of melon cultigens (Danesh *et al.*, 2015) used for organogenesis and transformation in this study.

Cultigen name	Area of origin	Group	Local name	Scientific name
'Eyvanaki'	Eyvanaki	Resembles Inodorus, but belongs to the Iranian cultivar group (Lotfi and Kashi, 1999).	Kharbozeh Eyvanaki	*Cucumis melo* 'Eyvanaki'
'Khatooni'	Mashhad	Resembles Inodorus, but belongs to the Iranian cultivar group (Lotfi and Kashi, 1999).	Kharbozeh Khatooni	*Cucumis melo* 'Khatooni'
'Samsoori'	Varamin	Cantalupensis	Talebi Samsoori	*Cucumis melo* 'Samsoori'

8 g l^{-1} agar. The pH of the medium was adjusted to 5.8 with 1 N KOH or HCl before autoclaving at 121 °C and 100 kPa for 15 min. Cultures were maintained at 25±1 °C under a 16 h photoperiod of 40-50 $\mu Em^{-2}s^{-1}$ light intensity provided by cool white fluorescent tubes, followed by 8 h of darkness.

Cotyledon, hypocotyl, and cotyledonary petiole (5 mm de-budded proximal cotyledon with its 2-3 mm-long hypocotyl stub) explants derived from 6-day-old *in vitro* grown seedlings were employed to determine the best explant type.

Plant regeneration

In order to study plant regeneration, a factorial experiment was conducted based on a completely randomized design (CRD) in four replications. The factors included four levels of BA (0, 0.5, 1, and 1.5 mg/L) for the induction of shoot, three melon cultigens, and three explant types (as explained above). Explants were incubated abaxial side down on MS medium supplemented with 0, 0.5, 1, and 1.5 mg l^{-1} BA for shoot induction. For shoot elongation, initiated shoots were cultured on an MS medium containing 0.1 mg l^{-1} BA plus 1 mg l^{-1} Gibberellic acid (GA_3). Finally, explants with a 2 cm shoot length were cultured on a growth regulator-free MS medium for root induction. The growth chamber condition was set up similarly to the seed germination condition. Treatments were replicated four times, and each replication (150×25 mm Petri dishes) included 10 segments. Explants were subcultured onto the same medium every three weeks after data collection. After rooting, plantlets with approximately 2 cm shoot length were transferred to the greenhouse and acclimatized within the plastic glasses containing autoclaved perlite and cocopeat (1:1 v/v) covered with transparent plastic lids for two weeks and subsequently transferred to the plastic pots containing perlite and cocopeat (1:1 v/v).

***Agrobacterium tumefaciens* inoculation and plant regeneration**

This experiment was conducted as factorial based on a completely randomized design with three factors including three strains of A. *tumefaciens* , inoculation time with four levels, 0.5, 1, 5, and 20 min, and co-cultivation time with three levels, 24, 48, and 72 h.

Avirulent *A. tumefaciens* strains including GV3101 (Radchuk *et al.,* 2000), LBA4404 (Hoekema *et al.,* 1983), and AGL0 (Lazo *et al.,* 1991) were employed for the genetic transformation of melon explants, carrying a binary plasmid vector pBL121 (Hoekema *et al.,* 1983). The pBL121 plasmid carries the *β-glucuronidase* (*GUS*) and *neomycin phosphotransferase* (*nptII*) as reporter genes in the T-DNA region, with CaMV 35S promoter and NOS terminator for constitutive transgene expression. These disarmed *A. tumefaciens* strains lacked the tumor-inducing (Ti) plasmid, but contained *vir* genes for transferring discrete DNA fragments (T-DNAs). Recombinant bacteria were cultured at 28 °C in a 50 ml liquid LB medium containing 50 mg l^{-1} kanamycin (Km) while shaking at 180 rpm. After about 12 h, when the final optical density (OD_{600} nm) reached 0.4 to 0.6, the bacterial suspension was centrifuged at 4 °C and 4000 rpm for 15 min. Finally, the pellet was resuspended in ½ MS medium containing 15 g l^{-1} sucrose, and the pH of the medium was adjusted to 5.8 for inoculation of explants.

Based on the previous tests, the cotyledonary petiole of 'Samsoori' was selected as an explant for the following procedure of transformation, selection, and regeneration. Therefore, the cotyledonary petiole explants were inoculated by soaking into the bacterial suspension for 0.5, 1, 5, and 20 min with gentle agitation. After inoculation, the explants were blotted dry and co-cultivated for 24, 48, and 72 h at 25±1 °C under darkness. After co-cultivation, the explants were washed with 200 mg l^{-1} cefotaxime (CTX) and transferred onto MS medium containing 100 mg l^{-1} Km plus 400 mg l^{-1} CTX, and 1 mg l^{-1} BA was used as a growth regulator, and the dishes were incubated under 16 h photoperiod. The explants were subcultured at 2-week intervals to the same medium for the selection of transgenic shoots. For shoot elongation, the selected Km-resistant putative transgenic shoots were transferred onto the MS medium containing 1 mg l^{-1} GA_3 plus 0.1 mg l^{-1} BA, 100 mg l^{-1} Km, and 400 mg l^{-1} CTX. Elongated green shoots with approximately 2 cm length were rooted on growth regulator-free MS medium without Km containing 400 mg l^{-1} CTX and samples of their leaves were taken for PCR and GUS assay. Each experiment, from infection to rooting was repeated four times to optimize the protocol. Finally, acclimatization of the explants was carried out as described in the plant regeneration section. All of the procedure was also performed using explants without *A. tumefaciens* infection and antibiotic treatment as the control.

Sensitivity to kanamycin

Sensitivity testing to Km was carried out to determine the dosage suitable for growth inhibition of non-transgenic sensitive tissues. Cotyledonary petioles were cultured on media containing different concentrations of Km including 0, 50, 75, 100, and 125 mg l^{-1} along with 400 mg l^{-1} CTX and 1 mg l^{-1} BA. Each treatment was replicated three times with 10 explants in each

replicate. Cultures were maintained at 25±1 °C and a 16 h photoperiod of 40-50 $\mu Em^{-2}s^{-1}$ light intensity provided by cool white fluorescent tubes, 8 h of darkness. After four weeks, the observations were recorded, and the data were analyzed based on a completely randomized design (CRD).

Polymerase chain reaction (PCR)

The genomic DNA of transformed and control plants was isolated from fresh young leaves using a modified CTAB method (De la Rosa *et al.,* 2002). In order to confirm the presence of the insert, PCR primers were used to amplify a 520-bp fragment from *GusA* comprising GUS-4 (5´-CCG GCA TAG TTA AAG AAA TCA TG-3´) and GUS-2 (5´-GGT GGT CAG TCC CTT ATG TTA CG-3´) as forward and reverse primers, respectively (Mousavi *et al.,* 2014). Also, specific PCR primers were used to amplify a 592-bp fragment from the *Agrobacterium Vir G* gene including VirG-FWD (5´-ATG ATT GTA CAT CCT TCA CG-3´) and VirG-REV (5´-TGC TGT TTT TAT CAG TTG AG-3´). The thermal cycler 480 (Perkin-Elmer, Foster City, CA) was used for amplification. The PCR cycles were run as follows: initial denaturation at 95 for 4 min, followed by 30 cycles of 94 °C (1 min), an annealing step at 55 °C (1 min), and 72 °C (1 min) and a final extension step at 72 °C for 5 min. The reaction mixture contained 2.5 µl 10X buffer (20 µM), $MgCl_2$ (3 µM), dNTPs (0.2 µM), forward and reverse primers each (0.2 µM), *Taq* DNA polymerase (0.2 µM), and template DNA (0.1 µg) bringing the total volume to 25 µl by double-distilled water. Genomic DNA extracted from non-transgenic melon plantlets was considered a negative control.

Transformation efficiency was evaluated as the number of PCR-positive plants concerning the initial number of explants cultured.

GUS histochemical analysis

In order to examine the expression of the *GusA* gene in putatively transformed plantlets, tissues were incubated in phosphate buffer, pH 8.0 containing chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) staining solution for 18 h at 37 °C followed by washing with 95% ethanol to remove chlorophyll as described by Mousavi *et al.* (2014) and then photographed.

Statistical analysis

The data was normalized using MiniTab software (Steel and JH Dickey, 1997) and evaluated using the Shapiro-Wilk statistic W test (Royston, 1992). The Box-Cox method (Box *et al.,* 2005) was used to transform the data. Each treatment was replicated four times with 10 explants in each replication. A factorial experiment in a completely randomized design with four replications was used to analyze the regeneration data, while a completely randomized design with five replications was used for the transformation data. Analysis of variance (ANOVA) using SAS (version 9.1) was conducted to test the data, and the means were compared using LS Means t-test at $P \leq 0.05$ to determine any significant differences.

RESULTS

Plant regeneration

Results of analysis of variance showed that the cultigens, BA concentration, and explant type had a significant ($p \leq 0.01$) effect on the percentage and number of regenerated shoots (Table 2). Meanwhile, the cultigens×BA concentrations, explant types×BA concentrations, and cultigens×explant type interactions were significant ($p \leq 0.01$) for the number of adventitious shoot production (Table 2).

Among tissues derived from seedlings of the same age, cotyledonary petiole explants showed the highest capacity of organogenesis or adventitious shoot induction with a maximum of 23 shoots per explant (Figure 1); particularly on MS medium with 1 mg l^{-1} BA for 'Samsoori' with an average of 19 shoots and with 1.5 mg l^{-1} BA for 'Khatooni' with 14 shoots per explant (Figures 1 and 2). The highest regeneration rate was obtained from 'Samsoori' followed by 'Khatooni' with an average of 17 and 14 shoots per explant, respectively (Figures 2 and 3).

Plant transformation

The results indicated that the shoot survival and growth rates were completely inhibited at 100 mg l^{-1} Km, and also showed the optimal threshold concentration for selection of transformants. Furthermore, the results showed that the rooting process was negatively affected by the presence of Km hence, this antibiotic was removed from the rooting media. Analysis of variance showed that *A. tumefaciens* strains, inoculation time, and co-cultivation time had a significant ($p \leq 0.01$) effect on the percentage of shoot regeneration and number of shoots per explants, whereas their interaction effects remained insignificant (Table 3). Comparing the three *A. tumefaciens* strains, with significant differences, the highest number of shoots per explants (0.62) was obtained from AGL0 followed by LBA4404 (0.38) and GV3101 (0.23), respectively (Figure 4). AGL0 not only had the strongest positive impact on regeneration system (Figure 4) but also displayed the highest transformation efficiency of

Table 2. Results of analysis of variance for the factorial experiment on effects of three explant types and four BA concentrations on shoot regeneration of three Persian melon cultigens. Data were backtransformed from the logarithmic average.

Source of variation	df	Mean of square	
		Percentage of shoot regeneration	Number of shoots per explant
BA concentrations	3	20352.55**	947.16**
Melons	2	3289.58**	655.46**
Explant types	2	27152.08**	1491.93**
BA concentrations×Melons	6	808.10^{ns}	188.70**
BA concentrations×Explant types	6	3142.82**	305.38**
Explant types×Melons	4	582.29^{ns}	198.11**
BA concentrations×Explant types×Melons	12	149.42^{ns}	73.62^{ns}
Error	108	111.34	8.66
Coefficient of variation (%)		5.85	5.33
R^2		0.85	0.91

*, **: Values significantly different at p<0.05and p<0.01, respectively, ns: non-significant.

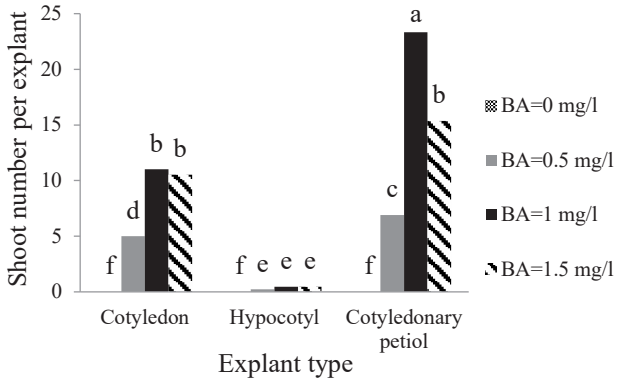

Figure 1. Mean comparison of explant types and BA concentrations on direct shoot regeneration. Means with different letters are significantly different at p<0.01.

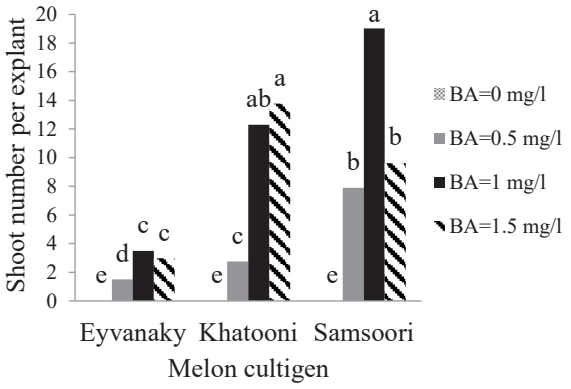

Figure 2. Mean comparison of melon cultigens and BA concentrations on direct shoot regeneration. Means with different letters are significantly different at p<0.01.

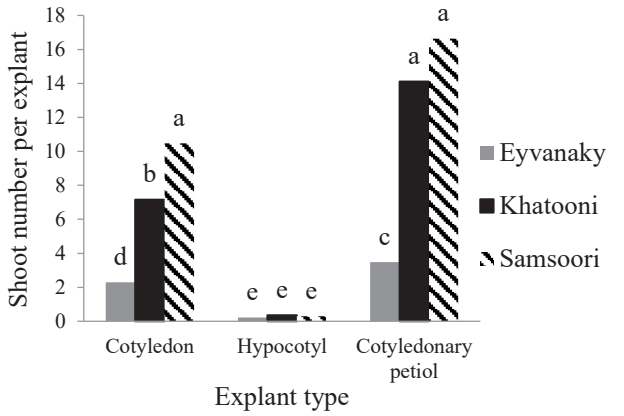

Figure 3. Mean comparison of explant types and melon cultigens on direct shoot regeneration. Means with different letters are significantly different at p<0.01.

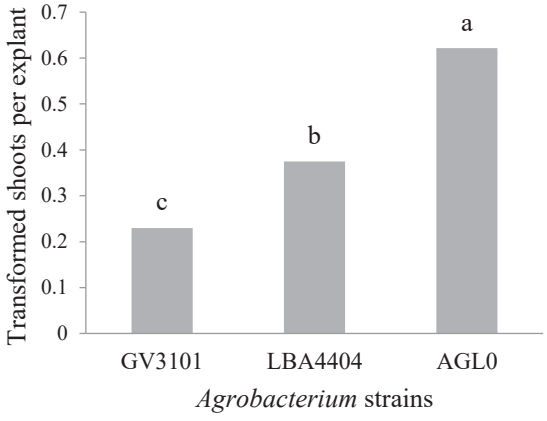

Figure 4. Effect of different *Agrobacterium* strains on direct shoot regeneration in each cotyledonary petiole explant of Samsoori melon. Means with different letters are significantly different at p<0.01.

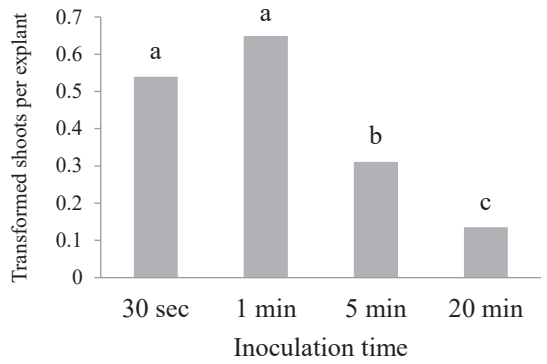

Figure 5. Effect of inoculation time on direct shoot regeneration in each cotyledonary petiole explant of Samsoori melon. Means with different letters are significantly different at $p<0.01$.

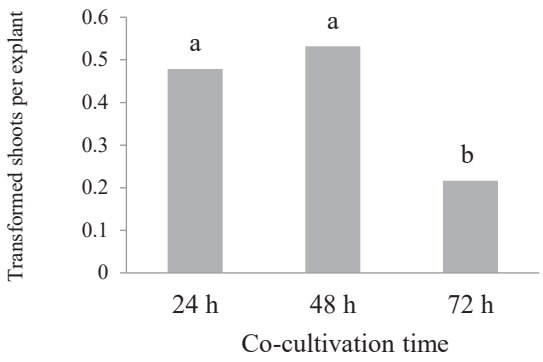

Figure 6. Effect of co-cultivation time on direct shoot regeneration in each cotyledonary petiole explant of Samsoori melon. Means with different letters are significantly different at $p<0.01$.

Table 3. Results of analysis of variance for the factorial experiment on effects of three *A. tumefaciens* strains, four inoculation times, and three co-cultivation times on transformation of *Cucumis melo* 'Samsoori'. Data were backtransformed from the logarithmic average.

Source of variation	df	Mean of square	
		Percentage of shoot regeneration	Number of shoots per explant
A. tumefaciens strains	2	1781.66**	2.35**
Inoculation time	3	2299.81**	2.38**
Co-cultivation time	2	1535**	1.70**
Inoculation time×*A. tumefaciens* strains	6	69.81^{ns}	0.27^{ns}
Co-cultivation time×*A. tumefaciens* strains	4	84.16^{ns}	0.15^{ns}
Co-cultivation time×Inoculation time	6	132.03^{ns}	0.26^{ns}
Co-cultivation time×Inoculation time×*A. tumefaciens* strains	12	24.53^{ns}	0.14^{ns}
Error	144	98.05	0.09
Coefficient of variation (%)		6.2	5.12
R^2		0.89	0.88

*, **: values significantly different at $p<0.05$ and $p<0.01$, respectively, ns: non-significant.

about 10%, which was followed by LBA4404 strain with 6%. Shoot multiplication was at the highest point (0.65) by exposure to one minute inoculation time, with no significant difference, followed by 30-second inoculation time (0.54) as the most effective duration for successful transformation (Figure 5). Concerning co-cultivation, 48 h exposure to co-cultivation medium induced the highest rate of shoot regeneration (0.53) and with no significant difference followed by 24 h (0.48) (Figure 6).

Histochemical GUS assay and PCR screening

Histochemical GUS assay verified the successful transformation of putative Km-resistant plantlets with blue coloration as a result of integration and expression of *GusA* transgene in transformants (Figure 7E). While the two-week-old transformed plantlets stained blue (Figure 7Ea), the leaves of control plants did not show any blue coloration (Figure 7Eb).

In this study, the plasmid pBL121 was used to transfer the *GusA* gene. Molecular analysis through PCR amplification confirmed the presence of the reporter gene (520-bp *gusA* fragment; Figure 8, lanes 4-16) as a result of successful transformation. The absence of the *Vir G*-specific band (592-bp fragment) verified the absence of *Agrobacterium* contamination in transformants, which stained blue in the GUS assay as a reliable indicator of true transgenic plants (Figure 9, lanes 4-15).

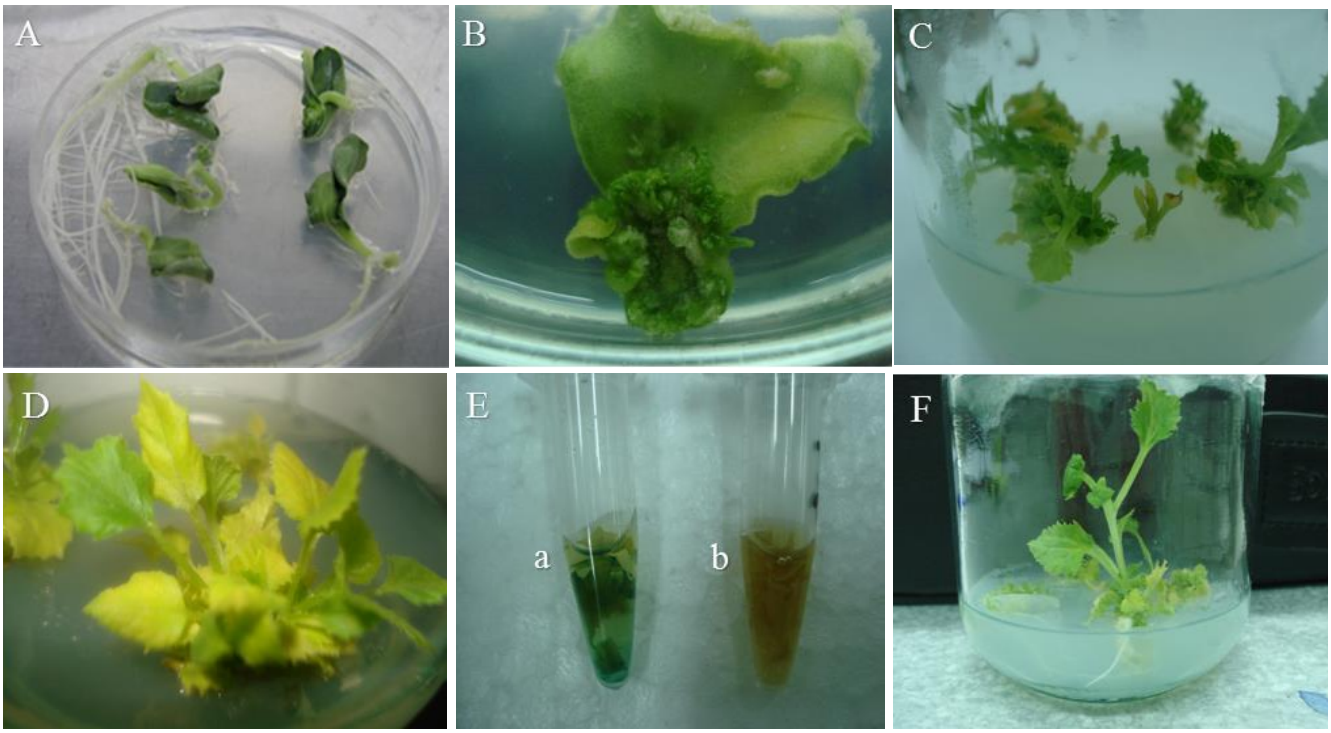

Figure 7. A: 6-day-old 'Samsoori' seedlings on ½ MS, **B:** Initiated buds from cotyledonary petiole of 'Samsoori' on MS medium plus 1 mg l^{-1} BA, 400 mg l^{-1} CTX, and 100 mg l^{-1} Km, three weeks after *Agrobacterium* inoculation, **C:** Transgenic kanamycin-resistant shoots on MS medium containing 1 mg l^{-1} GA_3, 0.1 mg l^{-1} BA, 400 mg l^{-1} CTX, and 100 mg l^{-1} Km, **D:** Non-transgenic and kanamycin-sensitive shoots on MS medium containing 1 mg l^{-1} GA_3, 0.1 mg l^{-1} BA, 400 mg l^{-1} CTX, and 100 mg l^{-1} Km, **E:** Differences in the histochemical staining pattern due to GUS activity between two-week-old young leaves of transformed *C. melo* 'Samsoori' (a) and control wild type plants (b) after 18 h at 37 °C under darkness, **F:** Rooting of 7-week-old Km resistant regenerants on growth regulator-free MS. medium.

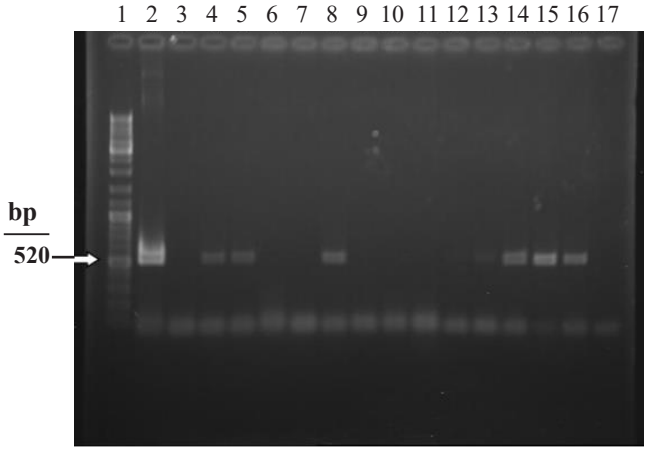

Figure 8. PCR analysis of genomic DNA of putatively transformed *C. melo* 'Samsoori' to detect the presence of *GusA* reporter gene. Lane 1 corresponds to 1 kb molecular size marker (Fermenats), lane 2 corresponds to positive control for pBI121 reporter gene, lanes 3 and 9 correspond to non-transgenic melon plantlets, lanes 6, 7, 10, and 11 correspond to non-transformed or escaped shoots, lanes 4, 5, 8, 12, 13, 14, 15, and 16 correspond to transformed plantlets, and lane 17 corresponds to the negative control (without transferred DNA).

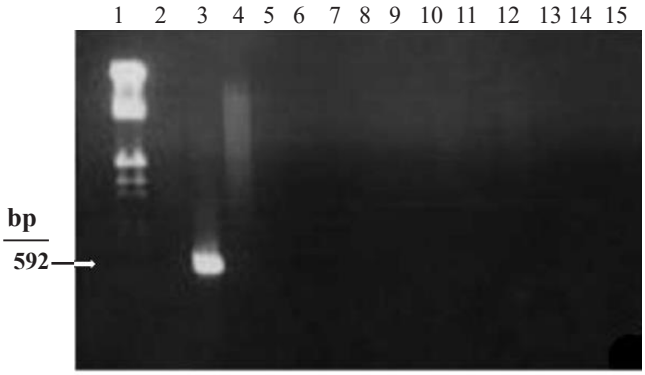

Figure 9. Molecular analysis of putatively transformed *C. melo* 'Samsoori' by PCR amplification with *vir G* primers to detect *Agrobacterium* contamination. *A. tumefaciens* genomic DNA was subjected to PCR with (lane 3, positive control) or without (lane 2, negative control) *vir G* primers. Lanes 4-15 contain total genomic DNA from putative transformants, indicating the absence of 592-bp *vir G* fragment; lane 1 corresponds to 1kb molecular size marker (Fermentas).

Most of the putative transgenic plantlets were rooted on growth regulator-free MS medium and around 90% of them successfully survived and acclimatized in the normal greenhouse conditions.

DISCUSSION

Plant regeneration

Despite developments in melon transformation and regeneration, low regeneration rate, long transformation procedure, and strong genotype dependency are considered the major hurdles to obtaining melons with superior organoleptic traits, higher yield, and disease resistance (Nuñez-Palenius *et al.,* 2008; Zhang *et al.,* 2014; Raji *et al.,* 2022).

Of the three types of tested explants (averaged over cultigen type and BA concentrations), the highest overall frequency of regeneration was obtained from cotyledonary petiole explants (Figures 1 and 3) showing consistency with previous reports (Pushyami *et al.,* 2011; Boszoradova *et al.,* 2011; Grozeva *et al.,* 2019) on the superiority of this explant over others. Compared with the cotyledon explant, regeneration from a proximal fragment of the cotyledon remaining attached to the hypocotyl stub is much more rapid and gives a higher percentage of explant regeneration. The lower regeneration capacity of melon cotyledons could be primarily attributed to the damage incurred at the end proximal of the cotyledon explant detached from the hypocotyl and the separation of the organs. Additionally, the absence or disruption in the translocation of growth factors from cotyledon to hypocotyl could restrict the regeneration capacity of the remaining hypocotyls. Finally and most importantly, the proximal end of hypocotyl contains young undifferentiated meristematic cells capable of initiating new shoot apical meristems after dissection (Curuk *et al.,* 2002; Grozeva *et al.,* 2019). It is botanically well-known that meristematic cells called meristemoids have a greater potential for bud induction.

Obtaining a procedure with a high regeneration frequency is a critical prerequisite for successful transformation. Using 1 mg l^{-1} BA for 'Samsoori' and 1.5 mg l^{-1} BA for 'Khatooni', bud and shoot production of cotyledonary petiole explants were rated the highest (by approximately 52% and 65%; not shown). The comparatively higher overall regeneration frequency and number of shoots per explant were obtained from cotyledonary petiole explants with 1 mg l^{-1} BA for 'Samsoori' and 1.5 mg l^{-1} BA for 'Khatooni' (Figures 1 and 2). A significantly higher percentage of cotyledonary petiole explants of 'Samsuri' and 'Khatoni' than 'Eyvanaky' initiated shoots (Figure 2). In addition to the explant sources, the organogenesis responses of melon species are highly genotype-dependant (Nuñez-Palenius *et al.,* 2008; Wan *et al*., 2023) and thus the *in vitro* regeneration conditions should be optimized based on genotype responses. In this regard, probably more combination of plant growth regulators is required to establish an efficient regeneration procedure for 'Eyvanaky'. This issue has been emphasized in other studies reporting lower rates of regeneration in melon cultivars (Grozeva *et al.,* 2019; Raji *et al.,* 2022).

Plant transformation

Similar to regeneration responses to growth regulators, plant sensitivity to antibiotics is species-dependent and exposure to antibiotics might exert a positive or negative influence on the regeneration system (Silva and Fukai, 2001; Wiebke *et al.,* 2006; Choi *et al.,* 2012). In our study, the explant survival rate and growth of all cultigens were completely inhibited at 100 mg l^{-1} Km, demonstrating the optimal concentration for selection of true transgenic plants. In other studies, this level of inhibition has been reported as 50 mg l^{-1} in Vedrantais cultivar (Akasaka-Kennedy *et al.,* 2004) and oriental melon (Choi *et al.,* 2012), and 200 mg l^{-1} in Silver light (Bezirganoglu *et al.,* 2014) and Charentais mono (Shirazi Parsa *et al.,* 2023).

Comparing the three *A. tumefaciens* strains, AGL0 consistently produced a significantly greater transformation response than LBA4404 and GV3101 at 0.4-0.6 OD_{600} (Figure 4). Strains LBA4404 (Guis *et al.,* 2000; Nora *et al.,* 2001; Bezirganoglu *et al.,* 2014; Raji *et al*., 2022) and EHA105 (Selvaraj *et al.,* 2010; Vengadesan *et al.,* 2005; Wang *et al.,* 2015) have been widely used for melon transformation with transformation efficiency up to 8.5% and 23%, respectively.

As shown in Figure 4, AGL0 was more efficient in inducing stable transformants by 10% transformation efficiency and 0.6 transformed shoots per explant markedly greater than LBA4404 with 6% transformation efficiency and 0.3 transformed shoots per explant (Figure 4).

Similar to the observations reported by Takavar *et al.* (2010) and Sutradhar and Mandal, (2023) where EHA101 and EHA105 strains (OD_{550} nm=0.4-0.5) caused extensive infection with a detrimental effect, GV3101 (OD_{600} nm=0.4-0.6) used in this study led to bacterial overgrowth that was not controlled with 200 or 400 mg l^{-1} CTX and drastically reduced the survival and transformation rates of explants (Figure 4).

A review of pertinent literature recommended more than 10 min inoculation time for preserving explant survival rate and regeneration capacity (Curuk *et al.,* 2005; Nonaka *et al.,* 2008; Nuñez-Palenius *et al.,* 2008; WANG *et al.,* 2015; Feng *et al*., 2023). In contrast, as shown in Figure 5, more than 5 min exposure to inoculation medium strongly reduced the survival and regeneration rates of explants from all cultigens, while 30 sec and 1 min treatments were found as the optimum time durations. A short inoculation time of 20 sec has been only once reported by Valles and Lasa (1994) in *Agrobacterium*-mediated transformation of *Cucumis melo* L., cv. Amarillo Oro.

The optimum co-cultivation duration of 24 h suggested in this study (Figure 6) has previously been proven to be beneficial in many types of research (Valles and Lasa, 1994; Curuk *et al.,* 2005; Chovelon *et al.,* 2008; Zhang *et al.,* 2014; Wang *et al.,* 2015; Li *et al*., 2020).

Knowing the fact that integration of T-DNA into plant genome takes more than 16 h (Agrawal and Rami, 2022), a co-cultivation time of less than 24 h may lead to a reduction of the transformation rate. Additionally, in co-cultivation longer than 48 h, antibiotics cannot control the infection causing a severe reduction of the survival rate and transformation capacity of explants. Hence, 24 h co-cultivation time was recommended as the optimum duration.

Co-cultivation at an ambient temperature of 25 °C led to the highest transformation efficiency, while temperature enhancement up to 29 °C resulted in bacterial overgrowth and consequently suppressed the survival rate and transformation of explants (data not shown).

Analysis of transgenic plants

First, histochemical analysis of transient GUS expression confirmed the transformation of *GusA* when two-week-old young leaves of transformed *C. melo* 'Samsoori' stained blue (Figure 7).

Next, the results of gPCR analysis corroborated the presence of *GusA* in Km-resistant transformants, indicating that the T-DNA of the binary plasmid vector was present in the genome of the transgenic plants (Figure 8). Last, the absence of *Vir G* fragment in transformants confirmed that the blue coloration of GUS assay was not related to *Agrobacterium* contamination (Figure 9).

CONCLUSION

By using cotyledonary petiole explants excised from 6-day-old seedlings, efficient plant regeneration via organogenesis and transformation was established for three Persian endemic melon cultigens 'Eyvanaki', 'Samsoori', and 'Khatooni'. The best regeneration frequency was obtained by applying 1 mg l^{-1} BA for 'Samsoori' and 1.5 mg l^{-1} BA for 'Khatooni', while the regeneration capacity of 'Eyvanaki' was substantially lower and required further studies to improve its regeneration rate. For transformation, the highest efficiency was obtained with *Agrobacterium* strain AGL0, 30 sec inoculation time, and 24 h co-cultivation time at 25 °C. Transformants, which resisted to 100 mg l^{-1} Km, were elongated on MS medium composed of 100 mg l^{-1} Km, 0.1 mg l^{-1} BA, 1 mg l^{-1} GA_3 plus 400 mg l^{-1} CTX and then successfully rooted on growth regulator-free MS medium during two weeks. The current transformation protocol which is optimized specifically for the Persian melons will facilitate the development of superior cultivars by introducing economically important genes such as improved postharvest life and resistance to biotic and abiotic stresses in this crop.

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