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REGENERATION AND AGROBACTERIUM-MEDIATED TRANSFORMATION OF WATERMELON

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ABSTRACT

In this study, a rapid regeneration system for two Egyptian watermelon cultivars (Giza 1 and Giza 21) was developed using the proximal zone of the hypocotyls. An *Agrobacterium*-mediated transformation system was also established for both cultivars. Results showed that the highest regeneration frequency of both cultivars (Giza 1 and Giza 21) was detected on explants that cultured on medium 5MSBAABA, as it revealed a percentage of 75 (Giza 1) and 72 (Giza 21) with 4-6 shoots/explants. It was also clearly observed that carbon source has an effect on the shoot regeneration frequency. Data showed that the hypocotyls explant revealed a regeneration percentage of 96.6 instead of 73.3 % when the sucrose was only used in 5MSAABA medium. Also, when 1.5g/L phytagel and 3g/L agar were used in 5MSBAABA medium the vitrification phenomenon of the developed shoots was decreased. A percentage of ~80 % of the shoots formed roots on the medium contained 40 µg/L NAA. At the level of transformation experiment, results showed that a number of 20 (16.7%) explants out of 120 explants of both watermelon cultivars were survived on the bialaphos- selection medium, and produced 42 (for Giza 1) and 33 (for Giza 21) bialaphos-resistant shoots. The presence of *gus-intron* (2070 bp) and *bar* (540 bp) genes was confirmed *via* polymerase chain reaction while their expressions were detected by histochemical GUS assay and leaf painting with 1 mg/L Basta herbicide.

INTRODUCTION

Watermelon (*Citrullus lanatus*) is an important vegetable crop worldwide with over 81 million metric tons produced annually. Egypt is considered the fourth country worldwide in producing watermelon (1.45 million metric tons) behind china (57.65 metric ton), Turkey (3.9 million metric tons) and Iran (1.9 million metric tons). Despite these high production figures, million of metric tons of fruit are lost in fields due to diseases infection. Genetic improvement through tissue culture and gene transfer plays an essential role in improving harvest fruits by of being with higher quality products or by introducing recombinant genes to improve biotic or abiotic stresses (Campton *et al.*, 2004). Establishment of the regeneration system is considered a critical stage of genetic transformation. Watermelon regeneration has been previously reported from a wide range of diploid and tetraploid cultivars using cotyledons of *in-vitro*-germinated seed-ling (Sirvasta *et al.*, 1989, Dong and Jia, 1991, Dong *et al.*, 1991,

Campton and Gray, 1993 and Hussein, 2003). Shoot regeneration from hypocotyl section has reported with a poor regeneration rate by Srivastara *et al*., (1989). In addition, Campton *et al*., (2004) used leaf segments as explants for producing shoots from watermelon. A novel direct shoot regeneration using the proximal zone of hypocotyl has efficiently applied in watermelon by Yalcin-Mendi *et al*., (2003).

In current study, efficient and rapid regeneration and transformation methods were established for the Egyptian watermelon cultivars, Giza 1 and Giza 21, using the proximal zone of hypocotyls.

MATERIALS AND METHODS

Plant materials: Watermelon seeds (*Citrullus lanatus*) cultivars Giza 1 and Giza 21 were obtained from the Horti-culture Research Institute, Agricultural Research Center (ARC), Giza, Egypt.

Plasmids and bacterial strains: The plasmid pISV2678 (Unpublished data) harboring *gus–intron uiA*d and *bar* genes (kindly provided by Institut des Sciences Vegetales (ISV), Centre National de la Recherche Scientifique (CNRS), Gif-Sur-Yvette, France) and *A. tumefaciens* strain LBA4404 (Horsch *et al*., 1985) were used in this work to establish plant transformation method.

Regeneration conditions: MS medium (Murashige and Skoog, 1962) was used in the regeneration and transformation experiments. The pH was adjusted at 5.8 before autoclaving. All plant cultures were incubated in controlled growth chamber at 25±2^{o}C under 8/16 h (dark/light) fluorescent lights.

Explants preparation: Watermelon pealed seeds were surface-sterilized and cultured in jars containing hormone-free MS medium and incubated for 7 days. Thereafter, the explants were prepared by excising the hypocotyls and dividing the cotyledons into two parts followed by removing the shoot apix by steilized scalpel, the proximal part of the cotyledon was stood on its hypocotyls stub.

Regeneration and acclimatization: To evaluate the regeneration ability of the hypocotyl explants, they were cultivated on MS based media with different concentration of plant growth regulations and $AgNO_3$ (Table 1) for 15 days

Table-1. Different shoot formation media composition.

Treatments	Media used			
	MSBA	5MSBA	MSBAABA	5MSBAABA
MS	+	+	+	+
BA	1mg/L	1mg/L	1mg/L	1mg/L
ABA	-	-	0.25 mg/L	0.25mg/L
AgNO3	-	5mg/L	-	5mg/L

Each experiment was repeated 3 times (with 90 explants each) with a total number of 270 explants. The produced shoots were excised from their original explants and cultured on rooting medium composed of MS medium with 40 µg/L

NAA as recommended by Hussein (2003) Subsequently rooted shoots were acclimatized into pots in a controlled greenhouse. The rest of explants, which did not produce shoots were transferred to a fresh regeneration medium.

Carbon source was also studied by adding different combinations of sucrose, fructose and glucose on 5MSAABA. To evaluate the effect of solidified agent on the watermelon regenerabilty, media

supplimented with agar and phytagel individually or/and together were tested.

***Agrobacterium*-mediated transformation system:** The explants of watermelon *cv* Giza 1 and Giza 21 were co-cultivated with *A. tumefaciens* strain LBA4404 harboring the plasmid pSIV2678. This was performed through two replicates and the numbers of explants in each replicate were 60 with a total number of 120 explants. The treated explants were cultured onto medium 5MSABA for 3 days. Subsequently, they were transferred to the selection medium (5MSBABA medium supplemented with 250 µg/L bialaphos and 300 mg/L carbinicillin) and incubated for 2-3 weeks. Developed shoots were excised from their original explants and then transferred to the root formation medium. Plantlets were then acclimatized in the greenhouse.

PCR assay: Genomic DNAs were isolated, as described by Dellaporta *et al.* (1983), from young leaves of putative transgenic plants and analyzed by PCR. Two pairs of specific primers were used to identify transformed plants, the first was designed to amplify 540 bp of the *bar* gene (namely **PI**, 5'AAA AGC TTC CAC CAT GAG CCC AGA ACG ACG3' and **P2**, 5'AAG GA T CCT TAG A TC TCG GTG ACG G') the second pair was designed to amplify the full *gus-intron* gene (2070 bp) (namely **P3**, 5'CCA GAT CTA ACA ATG CGC GGT GGT CAG TCC C3' and **P4,** 5'CCA GAT CTA TTC ATT GTT TGC CTC CCT GCT GC3'). The PCR temperature profile was as follows: initial denaturation of DNA at 94°C for 5 min, 35 cycles comprised of 1 min denaturation at 94°C, 1 min annealing at 55°C for *gus* gene or 60°C for *bar* gene, 1 min elongation step at 72 °C followed by a final extension step at 72 °C for 7 min.

Histochemical GUS and Leaf painting assays: Histochemical GUS assay was carried out on transformed explants and developed shoots. The tested tissues were immersed in 1 mg/mL 5-bromo-4-chloro-3-indolyl glucuroinde (X-gluc) for 24 h according to the CloneTech and incubated at 37°C over night. As negative control, non-transgenic tissues were tested. In order to evaluate the expression of *bar* gene, transformed watermelon leaves were painted with BASTA herbicide at concentration of 2 g/L.

RESULTS AND DISCUSSION

Watermelon regeneration: In this study, a rapid regeneration system of the Egyptian watermelon cultivars, i.e., Giza 1 and Giza 21 was developed using the proximal zone of the hypocotyls. An *Agrobacterium*-mediated transformation system was studied on the both cultivars.

The first visible reaction of the watermelon proximal zone explant, after few days of incubation, was the enlargement of these explants and the shoots regenerated directly during 2-3 weeks of culture. Data in Table-2 showed that the highest regeneration frequency of both cultivars (Giza 1 and Giza 21) was detected on explants that cultured on medium 5MSBAABA, as it revealed a percentage of 75 (Giza 1) and 72 (Giza 21) with 4-6 shoots/explants. The second best medium was MSBAABA, which revealed percentages of 65 and 63 for cvs. Giza 1 and Giza 2, respectively, with 3-4 shoots/explants, followed by medium 5MSBA which gave a percentage of 62 for Giza 1and 60 for Giza 21, with 3-5 shoots/explant. Finally, the lowest regeneration frequency was detected on medium MSBA representing percentages of 45 and 41 for the both applied watermelon cultivars (Giza 1 and Giza 21, respectively) with 3-4 shoots/explant. According to the previous data, it was observed that the high frequency of regeneration was obtained when the explants were cultured on

medium containing BA, ABA and silver nitrate ($AgNO_3$, 5 mg/L).

The proximal zone of hypocotyls has been successfully used before in water-melon (Yalcin-Mendi *et al.,* 2003), in melon (Curuk *et al.,* 2002 and Dessoky *et al*., 2006) and in tomato (Sheeja *et al*., 2004). The necessaries of adding BA for watermelon shoot formation has been previously reported by Compton and Gray (1993), Choi *et al.* (1994), Hussein (2003) and Yalcin-Mendi *et al.,* (2003). They obtained highest shoot formation on the medium containing BA ranging from 1-4 mg/L. In the current study, it was found that adding ABA increased the watermelon shoot formation frequency. It is the first time for successfully using ABA in watermelon regeneration although it was used successfully in melon (Niedz *et al.*, 1989; Fang and Grumet, 1990 and Hussein, 2003).

The promotive effect of $AgNO_3$ on *in vitro* shoot regeneration has been reported in melon by Yadav *et al*., (1996) using leaf explants and Hussien (2003) using cotyledon explants. They stated that the optimum regeneration frequency was obtained when $AgNO_3$ was added to the melon regeneration medium at concentration of 5 mg/L. However, thier was no improve in shoot regeneration has been observed with addition of $AgNO_3$ to melon or watermelon regeneration medium when cotyledonary explants (Niedz *et al*., 1989, Hussein, 2003) were used. The role of $AgNO_3$ was suggested by Beyer (1976, 1979) as that Ag^+ ion inhibits the ethylene action which does not interfere with C_2H_4 biosynthesis pathway, but with C_2H_4 incorporation at its sites of action. Ethylene's biosynthesis pathway comprises the conversion of S-adenosyl methionine into 1-aminocyclo-propane-1-carboxylic acid (ACC), under the action of ACC synthetase, and the conversion of ACC into ethylene, through the ethylene forming enzyme (EFE) or ACC oxidase (Zarembinski and Theologis, 1994).

Table 2: Frequency of watermelon regeneration on different media using the proximal zone of hypocotyls explant.

Parameters	Media Used							
	MSBA1		5MSBA1		MSBAABA		5MSBAABA	
	Watermlen cultivars							
	Giza1	Giza 21	Giza1	Giza 21	Giza1	Giza 21	Giza1	Giza 21
% Regeneration	45.8	41.2	62.7	60.3	65	63.1	75	72
% Vitrification	20	23	16.6	17.3	10.6	12	7	6
Shoots/explant	3-5	2-4	3-4	3-5	2-4	2-4	4-6	4-6

It was also clearly observed that carbon source is an important factor, which has an effect on the shoot regeneration frequency. Data recorded that the regeneration frequency increased when culturing the explants on medium containing equal volume of sucrose and glucose (15g/L of each). Table-3 shows that the hypocotyle explants revealed a regeneration percentage of 96.6 instead of 73.3 % when using 5MSAABA, which contains only sucrose. In addition, results showed that the explants cultured on medium containing equal volumes of sucrose and glucose did not obtain any vitrified shoots. Another factors had proved their efficiency in the

watermelon regeneration such as the solidified agents as well as subculturing for short period. Using medium 5MSBAABA, which contains 1.5g/L phytagel and 3g/L agar decreased the vitrification phenomenon of the developed shoots. In addition, the regeneration frequency increased when transferring the explants after 10 days to fresh medium (data not shown). Developed shoots were successfully rooted on medium containing 40 µg/L NAA. A percentage of ~80 % of the shoots forming roots was obtained during the first three weeks. Unrooted shoots were able to develop roots when transferring to the fresh rooting medium for another 2-3 weeks. Results in Figure-1 illustrate the regeneration steps, while those in Figure-2 illustrate the difference between the normal and vitrified shoots.

Table- 3: Effect of carbon source on the watermelon regeneration using the proximal zone of hypocotyls cv. Giza1.

Experiments	MC 30g/L	No. of total explants	No. of explants responded		No. of vetrifid shoots	
			No.	%	No.	%
Sucrose (S)	1	30	22	73.3	1	7
Fructose (F)	1	30	5	16.6	5	100
Glucose (G)	1	30	18	60.0	2	11
Sucrose/Fractose	1/1	30	12	40.0	1	8
	1/2	30	10	33.3	2	20
	2/1	30	13	43.3	2	15.4
Sucrose/Glucose	1/1	30	29	96.6	0	0
	1/2	30	12	40.0	1	8.3
	2/1	30	13	43.3	3	23
Fructose/Glucose	1/1	30	15	50.0	2	13.1
	1/2	30	9	30.0	1	11.1
	2/1	30	11	36.6	1	9
Sucrose/Fructose/Glucose	1/1/1	30	14	46.6	1	7
	2/1/1	30	15	50.0	0	0
	1/2/1	30	11	36.6	2	18
	1/1/2	30	14	46.6	3	21.4

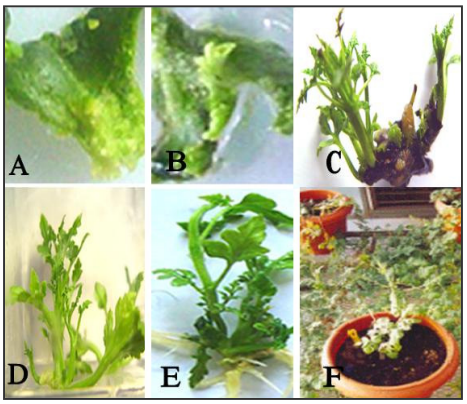

Figure-1: Regeneration stages of watermelon cv. Giza 1 using the proximal zone of hypocotyl as an explant; A) the proximal zone of hypocotyl explant after 5 days, B) shoot formation after 7 days, C) shoots developed from the explant after 3 weeks, D) developed shoots on rooting medium, E) Rooted plant after 3 weeks of culturing on rooting medium and F) acclimatized plant under greenhouse condition.

Figure-2*:* Normal watermelon shoots (Left) compared with vitirfied developed shoots (Right).

Watermelon transformation: Bialaphos was used as selectable marker for plant transformation. Results obtained from transformation experiments showed that the adventitious shoots started to appear after one week on the selection medium. These shoots reached 3-5 cm heighet within 2-3 weeks on the same mdium. A number of 20 explants out of 120 explants of both cultivars survived on the bialaphose medium, representing 16.7% responding explants. These explants produced 42 and 33 bialaphos-resistant shoots in cv. Giza 1 and cv. Giza 21, respectivily. Developed shoots were transferred on to the rooting medium for 3-4 weeks.

Evaluation of transformed plants with GUS and leaf painting assays: The bialaphos-resistant explants were randomly selected for detecting the expression of *gus* gene. Transformed explants developed blue color *via* GUS assay compared to non-transformed (remain white) as shown in Figure-3. All tested plant tissues developed blue color, indicating a correlation between GUS enzymatic activity and herbicide resis-tance, as all shoots were previously cultured on bialaphos-supplemented medium.

In addition, the bialaphos-resistant plantlets were painted with 1mg/L of Basta herbicide. The necrotic leaves which showed dark brown color were considered as negative (non-transgenic), while others which showed normal green color were considered as positive (trans-genic). Results in Figure-4, illustrate necrotic leave (untransformed) compared to transformed leave obtained after applying of BASTA.

Evaluation of transformed plants with PCR assay: The presence of both *gus*-intron and *bar* genes were confirmed on the bialaphos-resistant plantlets using the PCR technique with specific primers for each. Fragments of 2070 and 540 bp were amplified for *gus*-intron Figure-5 and *bar* Figure-6, respectively. Results showed that 38 plantlets out of 42 were positive for both genes in the case of Giza 1 while all the 33 were positive for both genes in Giza 21.

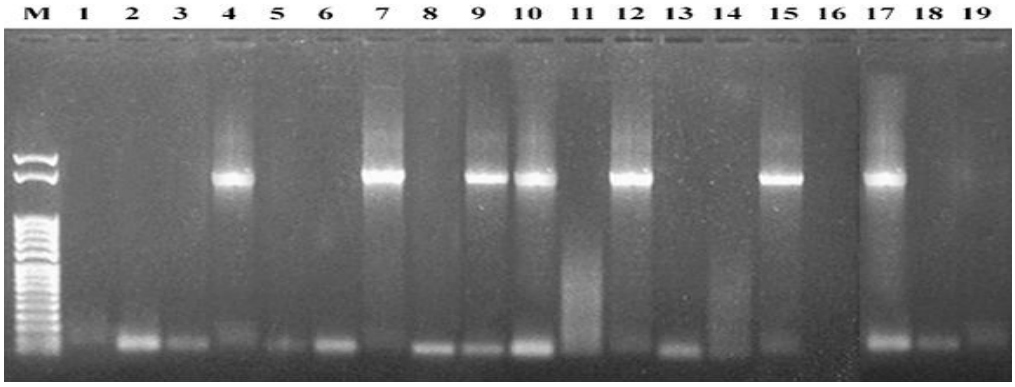

Figure -3: PCR detection of *gus-intron* gene into putative transgenic watermelon plants amplifiing 2070 bp with transgenic plants.

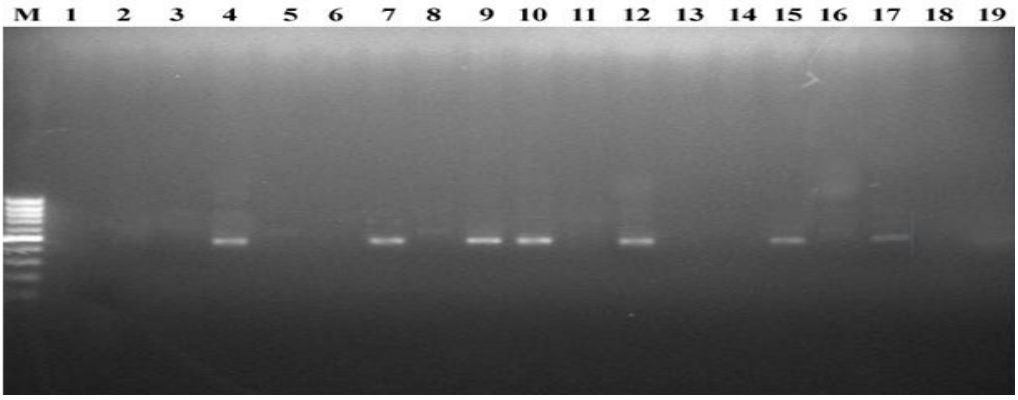

Figure-4: PCR detection of *bar* gene in putative transfgenic watermelon plants, amplifiing about 540 bp with transgenic plants.

Figure -5: Histochemical GUS assay of transformed hypocotyls with *Agrobacterium*.

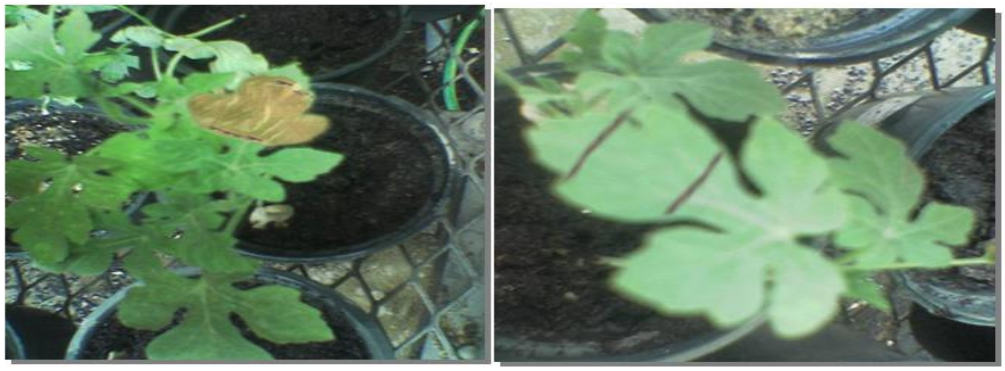

Figure-6: Leaf painting evaluation of transgenic plant (Right) compared to non-transformed plant (Left).

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