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# *IN VITRO* CALLUS INDUCTION AND SHOOT REGENERATION POTENTIALS IN SOME SNAKE MELON ACCESSIONS COLLECTED FROM DIFFERENT REGIONS IN EGYPT

**[177]**

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## ABSTRACT

Several snake melon genotypes are grown in different locations in Egypt. However, the relationships and the degree of relatness among these genotypes are not well documented. This study was carried out with the aim to classify different Egyptian snake melon genotypes based on the *in vitro* callus induction and shoot regeneration potential. Nine snake melon accessions (acc.) were collected from different regions in Egypt, including acc.3 (Damietta), acc.7 (Bany Swief), acc.8 (Fayoum), acc.9 (Giza), acc.11 (Menia), acc.14 (Sohag), acc. 15 (Behaira-Wady Elnatron), acc. 17 (Ismailia) and acc. 18 (Behaira-Badr Center). Twenty seeds from each accession were sterilized and cultured in vitro on MS medium basal salt and vitamins for 4 weeks. Segments (4x4mm each) from cotyledon were used as explants and cultured on MS medium amended with 2.0 mg/L TDZ for callus induction. The formed callus was sub cultured onto MS medium amended with 3.0 mg/L BA+4ml Hyaluronic acid for shoot regeneration. Results indicated that the degree of callus formation was different among the different accessions. Based on callus growth potential measured as fresh weight, snake melon accessions could be ranked as: acc.9> acc.18> acc.3= acc.11> acc.17> acc.15> acc.8= acc.14> acc.7. Significant differences among the accessions were also observed for their shoot regeneration potential from callus. The highest number of shoots per explant was recorded in acc. 18 (ave.12.6 shoots), followed by acc.9, 11, and 7 which produced an average of 10.8, 10.4, and 9.8 shoots, respectively. Results suggested that snake melon genotypes with high callus induction had also high regeneration capacity. In addition, these accessions could have different genetic background, which might help in future breeding programs to improve plant and agronomic traits. The current in vitro callus induction and shoot regeneration technique in snake melon will also aid in future effort for germplasm preservation of accessions with unique characteristics.

**Keywords:** *Cucumis melo* var. *flexuosus*, accessions, callus induction, shoot regeneration, Hyaluronic acid, TDZ.

## INTRODUCTION

Genotypic variation regarding the induction of morphogenetically competence cultures has been widely documented for several plant species. Such variations in the *in vitro* morphogenetic capacity were attributed to several factors such as explant source, physiological state of donor plant or culture conditions. However, several reports suggested that the induction of callus or organogenesis was under genetic control, and chromosomal location of the factors responsible for *in vitro* expression of morphogenesis has been identified early in wheat and rye **(Higgins and Mathias, 1987; Lazar et al 1987)**.

In **Chevrier et al 1990** found highly significant genetic differences for callus induction and regeneration of shoots in wheat, suggested that additive variance was an important factor in the inheritance of *in vitro* regeneration. In other study, **Ampormah-Dwamena et al (1997)** screened 22 lettuce genotypes for their response to regeneration in tissue culture and reported that shoot regeneration was strongly dependent on genotype. They showed no correlation between callus index

and shoot regeneration index. In addition, variation in the *in vitro* performance of the examined genotypes was not statistically linked to their morphological groups. The approaches utilized in their experiment enable the ranking and discrimination among genotypes based on the *in vitro* organogenic capacity.

In Cucurbits, highly significant differences in shoot forming capacity was reported among different genotypes of muskmelon **(Sebastiani and Ficcadenti 2016; Zhang et al 2011 and Ficcadenti & Rotino, 1995)**, *Cucurbita* spp. **(Gisbert et al 2011)**, and Cucumber **(Mohamed et al 2005)**.
The difficulty in introducing novel variability in melon by inter specific and inter generic hybridization pose great limitation to improvement by traditional breeding methods **(Nunez-Palenius et al 2008)**. Biotic methods such as genetic engineering, molecular biology and tissue culture techniques are capable of surpassing the natural genetic barriers, leading to the improvement of plant material and allowing the characterization of important horticultural traits.

Extensive screening of genotypes and modifying tissue culture conditions have greatly improved *in vitro* shoot regeneration of melon. In most research efforts, the focus was on testing factors affecting *in vitro* morphogenesis. However, limited studies have been directed to examine genotype differences. In melon, results of **Molina & Nnez (1995)** indicated that it was possible to detect genotypic variations when the genotypes differ by at least 10% in their regeneration frequency. Several other reports also pointed out the existence of genetic variability affecting regeneration ability between melon cultivars **(Blackmon & Reynolds, 1982; Bouabdallah & Branchard, 1986; Orts et al 1987; Mackay et al 1988, Dirks & Buggenum, 1989; Niedz et al 1989; Oridate et al 1992). Monlia and Nuesz (1995)** suggested that the existence of genetic variability in the *in vitro* melon regeneration could offer possibility of using this type of variation in future improvement programs.
Heredity models for *in vitro* regeneration were developed by several authors for melon in order to classify genotype based on their regeneration capacity or callus induction. In this respect, **Nadolska-Orezyk and Malepszy (1989)** classified genotypes on the basis of percent explants that developed embryogenic callus, and found a wide range of variation for the investigated trait within one genotype.

**Orts et al (1987)** classified 15 melon cultivars and accessions collected from different countries on the basis of percent calli with shoot buds and percent of calli with developed shoots *in vitro*. They found significant differences among the 15 genotypes and concluded that genetic variation in the *in vitro* shoot regeneration frequency would allow studies of the genetic control of this character. It was shown that six genotypes recorded more than 50% calli with shoot buds, while the percent calli with developed shoots was 44% in best, and 0.0% in the least genotypes. Similar findings were recorded by **Molina and Nuez (1995)** who examined variations in shoot regeneration capacity among seed population of *Cucumis melo* L. cv. Cantaloupe, and the study of **Ficcacdenti and Rotino (1995)** on eleven *Cucumis melo var. reticulatus* and *inodorus.*

Optimization of *in vitro* shoot regeneration in the family Cucurbitaceae was previously studied in muskmelon; winter squash **(Lee et al 2003)**; watermelon **(Compton and Gray, 1993)**; summer squash **(Ananthakrishnan et al 1993)**; bottle gourd **(Han et al 2005)**, and cucumber **(Curuk et al 2003)**. However, little studies have been conducted on the *in vitro* callus induction and plant regeneration in snake melon. The only available reports on this respect were those of **Yalcin-Mendi et al (2010a)** on a local Turkish genotype (46 KSU), and **Yalcin-Mendi et al (2010b)** on snake melon accession (Acur NEfe 34). The same Turkish research groups **(Comlekcioglu et al 2009)** have also reported on factors affecting somatic embryogenesis/embryonic callus induction from snake melon cotyledonary explants of un-known genotype. In the above mentioned articles, the focus was only directed to examine the best hormonal type and concentration, explant source and environmental conditions during culture incubation, and only one snake melon genotype, in each report, was used.

In their first trail, **Yalcin-Mendi et al (2010a)** examined several hormonal combinations (BA+IAA) and culture conditions for best shoot regeneration from cotyledonary segment explant. They found that the highest adventitious shoot regeneration rate (42.8) was obtained on MS medium supplemented with 1.0 mg/L BA+0.25 mg/L IAA using explants from dark-grown seedlings. No data were recorded on number of shoots regenerated per explants or the degree of callus formation. In their second study **(Yalcin-Mendi et al 2010b)** they obtained better shoot regeneration rate (88%)

from cotyledonary explants on MS medium supplemented with 0.5 mg/L BA+0.5mg/L IAA. Callus was formed on most explants on medium with BA. **Comlekcioglu et al (2009)** developed a protocol for *in vitro* regeneration by somatic embryogenesis in snake melon and reported the formation of callus on MS medium with high concentration of 2,4-D or NAA.

In the previous *in vitro* studies on snake melon, low BA concentrations were examined (0.25-2.0 mg/L) in combinations with IAA for shoot regeneration, or 2.4-D for callus induction. These plant growth regulators were not effective in our preliminary trail with the Egyptian snake melon accessions. However, results of **Keng and Hoong (2005)** on muskmelon showed high shoot regeneration capacity with high concentration of BA (8.0 mg/L) in the medium. Therefore, higher BA concentrations than those examined before and the use of TDZ (Thidiazuron) may need to be examined. TDZ was very effective in the regeneration of shoots from leaf disc in strawberry **(Mohamed et al 2007)**. It was also shown by **Gray (1993)** that TDZ stimulated the induction of somatic embryos in melon compared to BA, Kin, or 2ip. In addition, Hyaluronic acid (HA) was reported as new substance with high potential for *in vitro* shoot regeneration **(Kaewjampa et al 2012)** and need to be tried in snake melon tissue culture.

In the present study, variability among Egyptian snake melon accessions collected from different regions were examined based on their *in vitro* callus induction and regeneration capacity as a new approach to classify and seek possible relationship among the tested genotypes.

## MATERIAL AND METHODS

Variations among snake melon genotypes collected from different regions in Egypt were studied *in vitro* for both callus formation and shoot regeneration capacity based on the hypothesis that the genetic makeup of a plant genotype determines its *in vitro* performance in terms of callus induction and regeneration.

The plant materials included nine snake melon accessions, three from North Delta (acc. No.15, 3 and 18 which belong to Wady El-Natron, Behaira, Damietta, and Badr center, Behaira, respectively); one accession from middle delta (acc. No. 17 belong to Ismailia; five accessions from South Delta (8, 9,11,7 and14) belong to Fayoum, Giza, Menia, Bany Swif and Sohag, respectively.

### *In vitro* procedures

#### Callus induction experiment

Seeds from the different accessions were first sterilized by rinsing with tap water for 5 min, dipped in 70 Ethanol for 10 sec. followed by 5 min in 20% commercial bleach solution (Clorox) containing 1% NaOCl, then rinsed 3times in sterile distilled water. In a Laminar air-flow hood, seeds were cultured to germinate on a medium containing MS **(Murashige and Skoog, 1962)** basal salts and vitamins supplemented with 30g sucrose. Medium pH was adjusted to 5.7 before adding 7.0 g/L agar, then autoclaved for 30 min at 121ºC and 15 psi. Seeds were grown on the surface of the agar-solidified medium in a glass jars (ca.300 ml) as 5 seeds/jar. Cultures were incubated in a growth room at 24±2C$^{o}$ with 16 hr photoperiod under light of 60µmd $m^{-2}S^{-1}$. After 21 days from incubation, seeds were germinated into healthy seedling with fully developed cotyledons. The degree of callus formation among the different accessions was tested using cotyledonary explant segment (5X5 mm) from the middle of each cotyledon. Explants were cultured (abaxial surface up) on MS medium basal salts and vitamins amended with 30g/L sucrose and 2.0 mg/L TDZ or BA. Media were solidified with 7.0 g/L agar after adjusting pH to 5.7. Each accession was replicated 5 times (5 jars) containing 30 ml medium/jar, using three explants per jar. Cultures were incubated under dark condition for 2 weeks followed by light condition (16hr photoperiod, illumination of 45 µmol $min^{-2}S^{-1}$) for another 3 weeks. Cultures were arranged on the growth room shelves in a complete randomized design. Data were collected on callus proliferation potential by measuring callus fresh weight (g) and growth scale (1=small, 2=medium, and 3=massive callus). All cultured explants from the tested snake melon accessions have formed callus on MS medium supplemented with2.0 mg/L TDZ, but no callus was formed on MS+BA.

#### Shoot regeneration experiment

Calli formed from each accession were transferred to MS medium supplemented with 8.0 mg/L BA and 4 ml of HA acid to examine the difference among snake melon genotypes in their shoot regeneration capacity. Three calli were cultured per glass jar, each containing 30 ml medium. Five jars (replicates) were utilized per each accession and incubated on the shelves of the growth room in a

CRD design. After 6 weeks in culture, shoot regeneration potential was examined by measuring % of callus with developed shoots, number of shoots regenerated, regenerated cluster fresh weight (g), and number of fully developed buds.

**Statistical Analysis**

Data were subjected to the analysis of variance with means values compared with Duncan's multiple range test at 5% according to **Steel and Torrie (1980)**.

## RESULTS AND DISCUSSION

In the present study, callus and shoot /bud regeneration potential of nine snake melon accessions collected from different regions in Egypt were examined in a trial to rank these accessions and seek possible relationship based on the *in vitro* performance in two experiments.

In the first experiment, callus formation was achieved from the culture of cotyledonary explant on MS medium supplement 2.0 mg/L TDZ. Explants from all tested accessions had formed callus, mostly yellowish to white in color ranging from friable to compact callus. However, results revealed that the degree of callus formation was different among the different snake melon accessions as shown in **Table (1) and Fig. (2)**. In this regards, callus fresh weight (FW) was significantly higher in accession No.9 (4.6 g) and No. 18 (4.16 g). Accession No.3 and No.11 were similar in callus FW (3.88g), followed by acc. No.7, 17 and 15 **(Table 1)**. The least average callus FW was recorded in acc. No. 14. Similarly, callus rating tested in a scale from 1-3 indicated that acc. No.18, 17, 9, 11 and 7 were not significantly different and were the highest in callus mass than the rest of snake melon accessions *in vitro*.

Ranking scale is a qualitative trait, while measure of FW is a quantitative trait, therefore, ranking the performance based on the later measure could be more reliable than the former one. In this regards, the 9 snake melon accessions could be ranked as fellow: acc. No. 9>= 18>3= 11>7= 17>15>8=14. It is worth to mention that none of the cultured explants had formed callus on MS medium amended with 2.0 mg/L BA **(Fig. 1)** as compared to those on MS+ TDZ and this effect was true in all tested accessions except No.9 which showed little callus on medium supplemented with BA.

The above mentioned results indicated that all accessions examined had the potential to produce callus *in vitro*, irrespective of the source from which they were collected. However, the degree of callus formation showed significant differences among accessions. The acc. No.9 (from Giza) and No.18 (from Behaira, Badr) were almost similar in callus performance and showed best callus growth, while acc. No.8 (Fayoum) and No.14 (Sohag) recorded the least callus growth. It may be possible that the later accessions are genetically different than the former, especially being grown in greater distance from each other.

The good effectiveness of TDZ on the induction of callus from cotyledonary explant of snake melon in this study is the first to be reported, and went in agreement with other articles using different plant species, i.e. **Gondval *et al* (2016)** on medicinal herb and **Trivedi et al (2010)** on asparagus. It was reported by **Murthy et al (1998)** that TDZ has several effects *in vitro* since it exhibits the unique properties of both cytokinins and auxins.

In the second experiment for shoot regeneration, the subculture of callus onto medium supplemented with 8 mg/L BA+0.4% HA resulted in the formation of shoot buds after 4 weeks **(Figs. 3 and 4)**. It was found that all accessions had regenerated shoots on this medium, but in different degree, depending on the genotype **(Table 1)**. In this respect, the highest significant shoot cluster FW was recorded in acc.No.7 (5.53g), followed by acc. No.18 (5.17g) and acc. No.9 (4.98g). The least shoot cluster FW was found in acc. No.17 (3.64g). With respect to the number of regenerated shoots per explant, results revealed that acc. No.18 significantly produced the highest shoot number (12.6) followed by accessionsNo.9, 11 and 7 which produced10.8, 10.4, and 9.8 shoots/cluster, respectively. Adventitious shoot regeneration was significantly the least from callus of acc. No.15 (only 4.8 shoot/explants). Some accessions regenerated fully developed leaves from the shoot cluster with an average between 1.2 to1.4 leaf in acc. No.3, 9, and 7, as shown in **Table (1)**.

Based on the recorded number of regenerated shoots, the potential for shoot regeneration in the tested accessions could be ranked as follow: Acc. No.18 (Behaira, Badr) >Acc. No.9 (Giza = Acc. No.11 (Menia) = Acc. No.7 (BanySwif) > Acc. No.17 (Ismailia) = Acc. No.3 (Damietta) > Acc. No.14 (Sohag) > Acc. No.8 (Fayoum) > Acc. No.15 (Behaira, Wady El-Natron). These results suggest that some snake melon genotypes (No.18, 9, 11, and 7) had greater potential to regenerate shoots

**Table 1.** *In vitro* callus induction and regeneration capacity of nine Egyptian snake melon accessions

| Acc. * (No.) | Callus mass** | | Shoot regeneration*** | | Fully Developed leaf (No.) |
|---|---|---|---|---|---|
| | FW (g) | Rating Scale | FW (g) | Shoot/cluster (No.) | |
| 15 | 3.22 c | 2 b | 4.36 b | 4.8 e | 0.2 b |
| 3 | 3.86 b | 2 b | 4.08 bc | 8.8 bc | 1.4 a |
| 18 | 4.16 ab | 3 a | 5.17 ab | 12.6 a | 1.2 ab |
| 17 | 3.51 bc | 3 a | 3.64 c | 9.0 bc | 0.2 b |
| 8 | 2.85 d | 2 b | 4.24 b | 7.2 d | 0.2 b |
| 9 | 4.26 a | 3 a | 4.98 ab | 10.8 b | 1.4 a |
| 11 | 3.88 b | 3 a | 4.55 b | 10.4 b | 0.2 b |
| 7 | 3.52 bc | 3 a | 5.53 a | 9.8 b | 1.4 a |
| 14 | 2.66 d | 2 b | 4.10 bc | 8.2 c | 0.2 b |

Callus rating:1= small, 2= medium, 3= massive callus.

* See materials and methods for the name and region of each accession.

** Callus induction on MS+2.0 mg/L TDZ.

*** Shoot regeneration on MS+8.0 mg/L BA+0.4%HA.

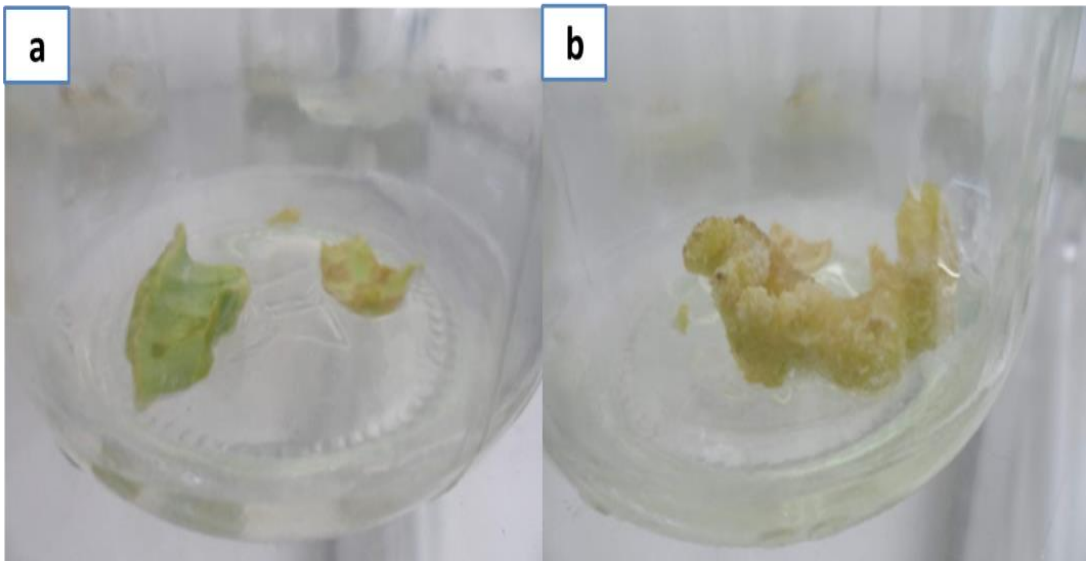


**Fig. 1.** No callus formation from snake melon explants on MS+2.0 mg/L BA (a), and little callus from explants of accessions No.14 on the same medium

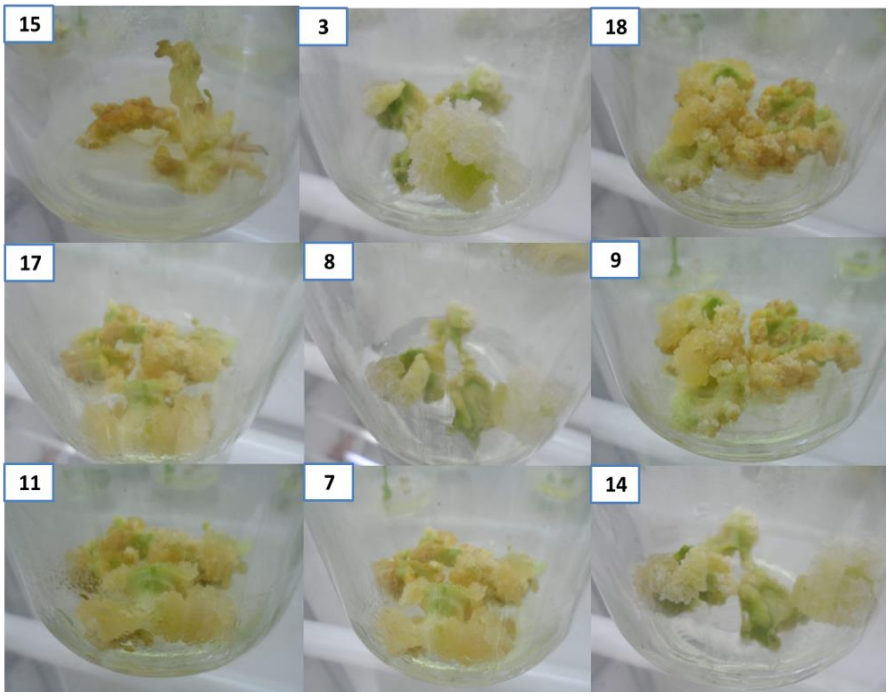


**Fig. 2.** Callus induction from cotyledonary explants of different Egyptian snake melon accessions. Accession No.15,3,18,17,8,9,11,7 and 14 were collected from Behaira (Wady-Natron), Damietta, Behaira (Badr), Ismailia, Fayoum, Giza, Menia, Bany Swif and Sohag, respectively.

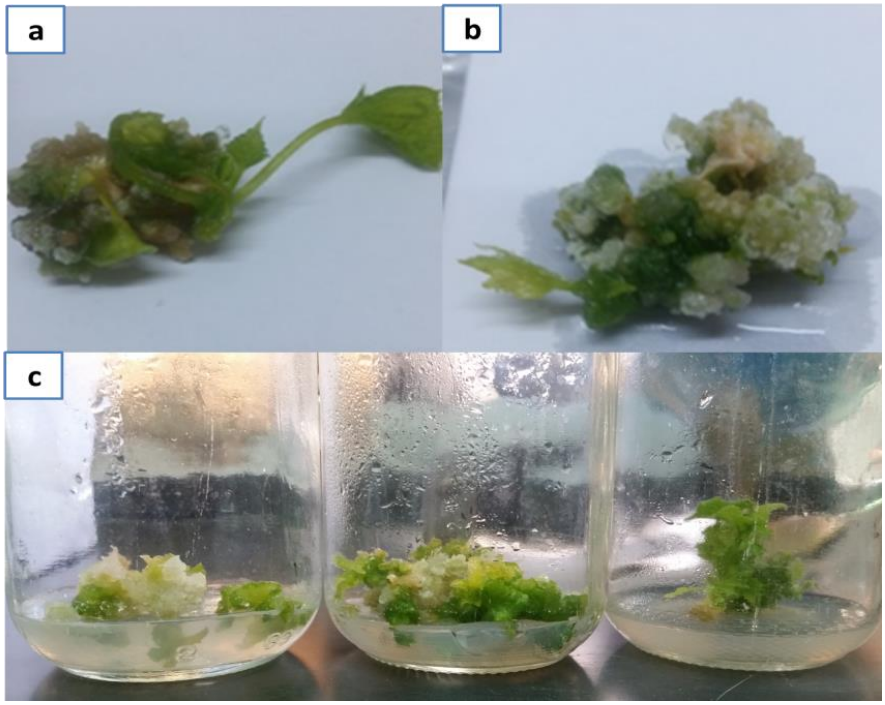


**Fig. 3.** Fully developed snake melon shoots from regenerated callus (a and b); developmental stages of regeneration from callus (left jar), regenerated cluster (middle) and fully developed shoots (right) jar (c).

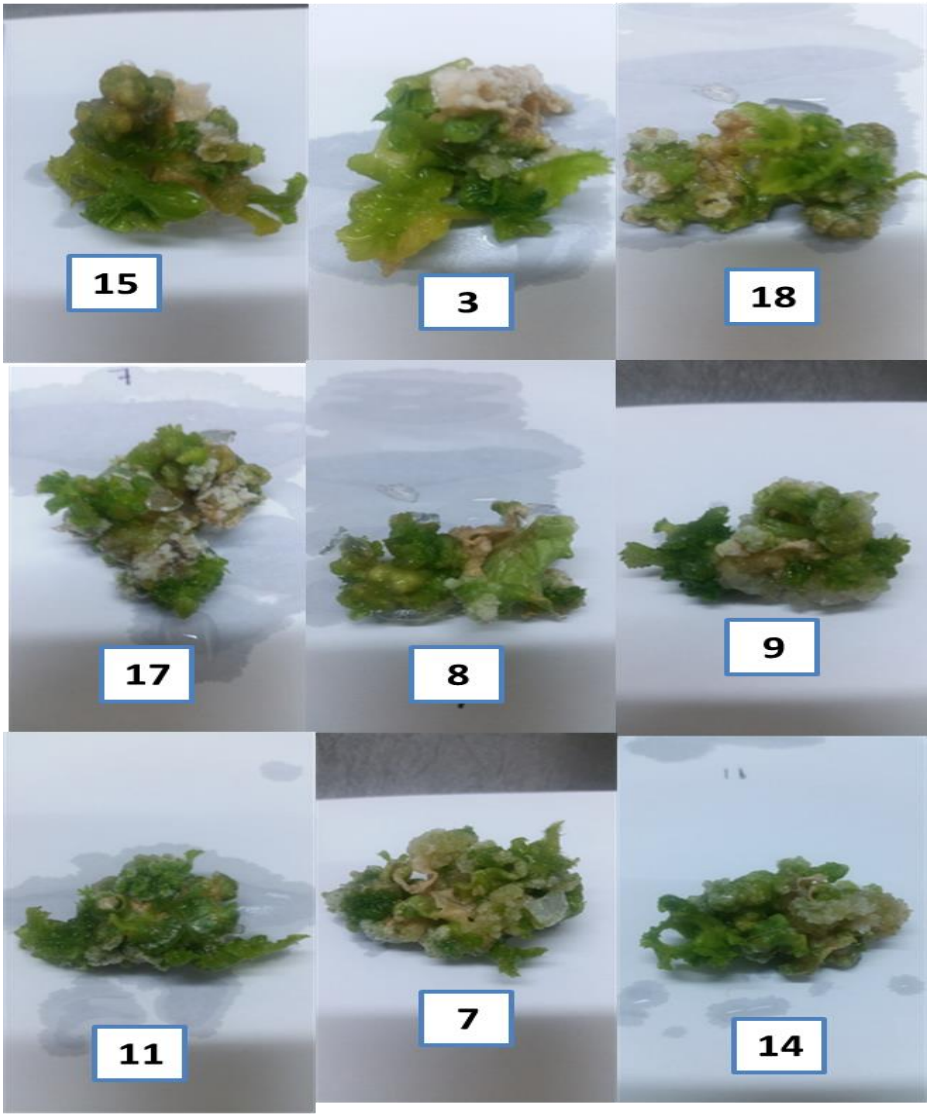


**Fig. 4.** *In vitro* shoot regeneration from callus of different snake melon accessions on MS+8.0 mg/L BA+0.4% HA. Accessions No. 15, 3,18,17,8,9, 11, 7 and 14 were collected from Behaira (Wady El-Natron), Damietta, Behaira (Badr), Ismailia, Fayoum, Giza, Menia, Bany Swif and Sohag, respectively

*in vitro* and almost had the same potential for callus induction. Other genotypes (No.15, and 8) had the lowest shoot regeneration capacity. It was also obvious that callus and shoot regeneration in the tested snake melon genotypes did not depend on the location from which they were collected. In this regards, accessions with the highest shoot regeneration were acc. No.7 from Bany Swif, acc. No.18 from Behaira, and acc. No.9 from Giza, all were collected from wider distances from each other. In the two *in vitro* experiments reported herin, all factors of *in vitro* culture (explants source and size, medium components and incubation conditions) were similar and the only variable was the snake melon genotype. However, significant differences exist among these genotypes in their totipotent nature to from callus and regenerate shoots *in vitro*. If these differences were due to their different genetic makeup, one might assume that accession with high *in vitro* growth potential (acc.No.9, and 18) or those with low potential (acc. No.15, 8, 14) might be closely related. In fact, ISSR analysis indicated that, most genotypes examined had close genetic similarity **(Mohamed et al 2019)**. In

accordance with the results of **Molina and Nuez (1995)** in melon, it was possible to detect genotypic variation when the genotypes differ by at least 10% in their regeneration ability. The existence of genetic variability affecting the regeneration among melon genotypes was also reported in several studies **(Mackay et al 1988; Dirks & Buggenum, 1989; Niedz et al 1989; Molina & Nuez, 1995, and Ficcadenti & Rotino, 1995)**.

The stimulatory effect of the cytokinin (BA) on the *in vitro* shoot regeneration of snake melon in our study is well documented according to the study of **Yalcin-Mendi et al (2010 a,b) and Comlekcioglu et al (2009)**. However, they used lower concentration of BA in the medium (0.25-2.0 mg/L) than that used in the present study (8.0 mg/L).Perhaps the different response to BA concentration could be due to differences in the genetic background between the Turkish and Egyptian snake melons. In addition, due to the high level of BA in combination with the addition of HA to the culture medium (as a new plant growth regulator), high number of regenerated shoots from the different snake melon accessions was achieved. These results are in harmony with those of **Kaewjampa et al 2012** in micro propagation of hybrid cymbidium, and **Hefni, 2018** on shoot regeneration of *Cucuma longa* using HA in the medium. The functions of hyaluronic acid (HA) include regeneration of protein secretion, gene expression, cell proliferation and differentiation **(Fraser et al 1997)**.

In conclusion, this study demonstrated for the first time the induction of callus on TDZ-amended medium and shoot regeneration on MS medium supplemented with 8.0 mg/L BA+0.4 HA in different Egyptian snake melon genotypes. This protocol allowed ranking snake melon accessions collected from different regions in Egypt based on their callus and shoot regeneration potential under *in vitro* condition. Shoot regeneration protocol is considered the first step in any genetic engineering program and the utilization of biotechnology for the improvement in snake melon plants. Another advantage of our protocol is the possibility of introducing new trait (high regeneration capacity) into the genotype of low regeneration potential. Finally, *in vitro* micro propagation system can be a useful tool for germplasm preservation *in vitro* of snake melon grown in Egypt to be used in future breeding programs. Somaclonal variations among regenerated plant in snake melon are another tool for future studies in their breeding strategy.

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# كفاءة تكوين الكالوس وتجديد التبرعم الخضري معمليا في بعض سلالات القثاء المجمعة من مناطق مختلفة في مصر

# [177]

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استهدفت الدراسة تقسيم بعض الأصول الوراثية لنبات القثاء بناء على القدرة على تكوين الكالوس وتجديد التبرعم الخضري بمزارع الأنسجة حيث تم جمع تسعة سلالات من مناطق مختلفة من جمهورية مصر العربية تضمنت محافظة البحيرة– وادي النطرون (سلالة 15) والبحيرة– مركز بدر (سلالة 18) ودمياط (سلالة 3) والجيزة (سلالة 9) والمنيا (سلالة11) وبني سويف (سلالة 7) والاسماعيلية (سلالة 17) وسوهاج (سلالة 14) والفيوم (سلالة 8). تم زراعة قطع من الأوراق الفلقية على بيئة موراشيج وسكوج المضاف اليها 2 مجم في اللترثيادوزورون لتكوين الكالوس ثم تم نقل الكالوس المتكون الي بيئة مضاف اليها 8 مجم /لترهرمون بنزيل أدينين و 4 % حمض الهيلورونيك لتكوين التبرعم الخضري. أوضحت النتائج اختلاف في درجة تكوين الكالوس بين السلالات المختلفة وأمكن ترتيب السلالات حسب الوزن الطازج للكالوس من الاعلي الي الأقل كالتالي : السلالة رقم 9 ثم رقم 18 ثم رقم 3 التي تساوي رقم 11 الأكبرمن رقم 15 ثم رقم 8 وهي أكبرمن او تساوي السلالة رقم 14. كما وجدت فروق معنوية بين السلالات في القدرةعلي التبرعم الخضري حيث أعطت السلالة رقم 18 أعلى عدد من البراعم الخضرية بمتوسط 12,6 برعم من كتلة الكالوس تليها السلالات رقم 9 و11 و7 والتي أنتجت متوسط 10,8 و 10,4 و 9,8 برعم علي الترتيب. بينما وجد أن أقل السلالات في التبرعم الخضري كانت السلالة رقم 15 بمتوسط 4,8 برعم. ويقترح من تلك الدراسة أن سلالات القثاء ذات القدرة العالية على تكوين الكالوس تعتبر أيضا ذات قدرة عالية علي تجديد التبرعم الخضري بمزارع الأنسجة إضافة الي أن هذه السلالات قد تختلف في تكوينها الوراثي مما يساهم في برامج التربية في هذا المحصول.

**الكلمات الدالة:** القثاء، احداث الكالوس، التبرعم الخضري، حمض الهيلورونك، ثيادوزورون

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