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

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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 Elinatron), 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, Ampornmah-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 \textit{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 \textit{in vitro} organogenetic capacity.

In Cucurbitaceae, highly significant differences in shoot forming capacity were reported among different genotypes of muskmelon (Sebastiani and Ficcadenti 2016; Zhang et al 2011 and Ficcadenti & Rotino, 1995). \textit{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-Paleniuss 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 \textit{in vitro} shoot regeneration of melon. In most research efforts, the focus was on testing factors affecting \textit{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, 1996; Orts et al 1987; Mackay et al 1988, Dirks & Buggenum, 1989; Niedz et al 1989; Oridate et al 1992). Monila and Nuesz (1995) suggested that the existence of genetic variability in the \textit{in vitro} melon regeneration could offer possibility of using this type of variation in future improvement programs. Heredity models for \textit{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 \textit{in vitro}. They found significant differences among the 15 genotypes and concluded that genetic variation in the \textit{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 \textit{Cucumis melo} L. cv. Cantaloupe, and the study of Ficcadenti and Rotino (1995) on eleven \textit{Cucumis melo} var. \textit{reticulatus} and \textit{inodorus}.

Optimization of \textit{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 \textit{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 cotyledonal 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 trial 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 2Zip. In addition, Hyaluronic acid (HA) was reported as a 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 in Egypt were studied 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 121°C and 15 psi. Seeds were grown on the surface of the agar-solidified medium in a glass jars (ca.300 ml) at 5 seeds/jar. Cultures were incubated in a growth room at 24±2°C with 16 hr photoperiod under light of 60µmd m⁻²S⁻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 cotyledon 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⁻²S⁻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 with 2.0 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
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 cotyledonal explant on MS medium supplemented 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 cotyledonal 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 accessions No.9, 11 and 7 which produced 10.8, 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/expplants). Some accessions regenerated fully developed leaves from the shoot cluster with an average between 1.2 to 1.4 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, 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).
In vitro callus induction and shoot regeneration potentials in some snake melon accessions collected from different regions in Egypt

AUJASCI, Arab Univ. J. Agric. Sci., 27(4), 2019

**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 *vivo* 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), a 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 cumbidium, and Hefni, 2018 on shoot regeneration of Cucumis 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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Zhang H., Peng G. and Feishi L. 2011. Efficient plant regeneration from cotyledonal node explants of *Cucumis melo* L. *African J. Biotechnol.*, 10(35), 6757-6761.
The study aimed to classify some of the genetic lines of pearling pearl using the ability to form callus and induce leaf bud regeneration in tissue culture. Tissue culture was conducted in a medium containing Murashige and Skoog supplemented with 2 mg/l Thidiazuron and 1% Hyaluronic acid for callus formation, while the shoot regeneration was carried out in a medium containing 1 mg/l NAA and 1% BAP. Nine lines from different areas in Egypt, including: line No. 11 from Faiyum, line No. 15 from Al-Badri, line No. 17 from Assiut, line No. 9 from Qena, line No. 3 from Damietta, line No. 7 from Beni Suef, line No. 11 from Minya, line No. 11 from Qena and line No. 11 from Qena. The results showed significant differences in the ability to form callus between the different lines, and it was possible to rank the lines according to the dry weight of the callus from highest to lowest as follows: line No. 9 then No. 11 then No. 3 which is equal to No. 11 the highest than No. 11 then No. 1 and is greater than or equal to line No. 11. In addition, there were significant differences between the lines in the ability to induce leaf bud regeneration where line No. 11 gave the highest number of green sprouts of an average of 12.21 sprouts per callus bunch, followed by lines No. 9 and 11 and 7 and 1921 sprouts respectively. While it was found that the least lines in leaf bud regeneration were line No. 11 with an average of 121 sprouts. It is recommended that lines of pearling pearl with high ability to form callus be also with high ability to induce leaf bud regeneration in tissue culture, in addition to these lines may differ in their genetic constitution, which will be a basis for breeding programs in this crop.

Key words: Pearling pearl, Induction of callus, Leaf bud regeneration, Hyaluronic acid, Thidiazuron.