تأثير منظمات النمو ومكونات الوسط المغذي في الإكثار المخبري للعنب (*Vitis vinifera* L.) صنف "مطروح أسود"

Effect of Plant Growth Regulators and Medium Constituents on *In Vitro* Propagation of Grape (*Vitis vinifera* L.) cv. "Black Matrouh"

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الملخّص

تم تنفيذ هذا البحث خلال الفترة من 2011 إلى 2013 في مخبر زراعة الأنسجة في البنك القومي للجينات والمصادر الوراثية في جمهورية مصر العربية، بهدف إيجاد طريقة فعالة للإكثار الدقيق للعنب (*Vitis vinifera* L.) صنف "مطروح أسود" باستخدام القمم النامية والعقد الساقية كعزلات نباتية. زُرعت العزلات النباتية على وسط موراشيج وسكوج (MS) بكامل تركيز الأملاح المعدنية، ثلاثة أرباع التركيز (MS¾) أو نصف تركيز الأملاح المعدنية (MS½) مدعماً بالبنزيل أمينو بيورين (BAP) بتراكيز 0.2، 0.5، 1.0 و 1.5 مغ/ل في مرحلة التأسيس.

في مرحلة الإكثار، تم اختبار وسط MS بكامل التركيز أو ثلاثة أرباع تركيز الأملاح المعدنية (MS¾) مدعم بـ BAP بتراكيز 0.50، 0.75 و 1.00 مغ/ل لوحده أو مع إندول حمض الزبدة (IBA) بتراكيز 0.10، 0.30 و 0.50 مغ/ل. تم في مرحلة التجذير اختبار تأثير نوعين من الأكسين؛ IBA ونفتالين حمض الخل (NAA)، بتراكيز 0.1، 0.3، 0.5 و 1.0 مغ/ل في وسط MS بنصف قوة الأملاح المعدنية (MS½). خلال هذه الدراسة، أظهرت القمم النامية والعقد الساقية لنبات العنب أعلى نسبة بقاء على وسط MS بثلاثة أرباع تركيز الاملاح المعدنية مع 0.5 مغ/ل BAP (100 % و 88.89 %، على التوالي). لوحظ أعلى عدد أفرخ/القمة النامية (1.86) على وسط MS بتركيز كامل من الأملاح المعدنية مع 0.5 مغ/ل BAP، بينما تم إنتاج أعلى عدد أفرخ/ العقدة الساقية (1.76) على وسط MS بكامل تركيز الأملاح المعدنية مع 1.0 مغ/ل BAP. سُجل أعلى عدد أفرخ/ العزلة النباتية من مرحلة التأسيس والمزروعة على وسط MS بثلاثة أرباع تركيز الأملاح مع (0.75 BAP+5.0 IBA)مغ/ل أو (1.00 BAP+3.0 IBA)مغ/ل (3.67 و 3.52 فرخ/عزلة، على التوالي)، بينما أنتجت العزلات الناتجة من مرحلة التأسيس والمزروعة على وسط MS بكامل تركيز الأملاح والخالي من منظمات النمو أطول الأفرخ (4.94 سم). بشكل عام كانت نسبة التجذير أعلى للأفرخ المزروعة على وسط MS بنصف تركيز الأملاح ومدعم بالأكسين بالمقارنة مع الوسط الخالي من الأكسين. سُجل أعلى عدد للجذور/الفرخ (5.50 جذر/فرخ) على وسط MS بنصف تركيز الأملاح مع 0.5 مغ/ل NAA وباستطالة مقبولة (4.11 سم) دون فروق معنوية مع أعلى استطالة للجذور (5.06 سم)، والتي سُجلت على وسط MS بنصف تركيز الأملاح مع 0.5 مغ/ل IBA .

الكلمات المفتاحية: العنب صنف "مطروح أسود"، الإكثار الدقيق، قمة نامية، عقدة ساقية، وسط MS، BAP، تجذير، NAA، IBA.

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Abstract

This study was carried out during the period 2011- 2013 at the laboratory of Tissue Culture in the National Gene Bank and Genetic Resources, Egypt; in order to obtain an efficient method for micropropagation of grape (*Vitis vinifera* L.) cv. "Black Matrouh". The explants were cultured on full, three-quarter or half strength Murashige and Skoog (MS) medium supplemented with 6-benzylamino purine (BAP) at 0.2, 0.5, 1.0 or 1.5 mgl^{-1} for establishment stage. For multiplication stage, full or three-quarter strength MS medium supplemented with BAP at 0.50, 0.75 or 1.00 mgl^{-1} alone or in combination with indol-3-butyric acid (IBA) at 0.10, 0.30 or 0.50 mgl^{-1} were tested. Rooting stage was investigated on half strength MS medium supplemented with IBA or naphthalene acetic acid (NAA) at 0.1, 0.3, 0.5 or 1.0 mgl^{-1}. During the present study, grape shoot tip and nodal explants showed the highest survival percentages on three-quarter strength MS medium with 0.5 mgl^{-1} BAP (100% and 88.89%, respectively). The highest number of shoots/shoot tip (1.86) was noticed on full strength MS medium with 0.5 mgl^{-1} BAP, while full strength MS medium with 1.0 mgl^{-1} BAP produced the highest shoot number/nodal cutting (1.67). Established explants cultured on three-quarter strength MS medium with (0.75 BAP+0.50 IBA) mgl^{-1} or (1.00 BAP+0.30 IBA) mgl^{-1} produced the highest shoot number/explant (3.67 and 3.52 shoots/explant, respectively), while established explants cultured on growth regulator-free full strength MS medium showed the highest shoot length (4.94 cm). In general, the rooting percentage was the highest on half strength MS medium with auxin compared to those on auxin-free medium. Half strength MS medium with 0.5 mgl^{-1} NAA exhibited the highest roots number/shoot explant (5.50 roots/shoot) with adequate root length (4.11 cm) without significant difference compared to the longest roots (5.06 cm) which were observed on half strength MS medium with 0.5 mgl^{-1} IBA.

Key word: Grape cv. "Black Matrouh", Micropropagation, Shoot tip, Nodal cutting, MS medium, BAP, Rooting, IBA, NAA.

Introduction

Grape (*Vitis vinifera* L.) is an old deciduous temperate fruit crop under the family Vitaceae, widespread and highly valuable because of its nutritional characteristics as a natural source of sugar, vitamins and fibers (Gomes et *al*., 2004). The conventional method of grape propagation is time consuming and allows diseases transmission. By using the tissue culture technique, a mass production of genetically homogeneous populations and healthy plants occurs. Therefore, it is a very important technique for grape culture program (Kinfe, 2010). Grape can be easily and rapidly propagated in vitro from shoot tips and axillary buds (Harris and Stevenson, 1982) maintaining its genetic stability.

In vitro multiplication of plants depends on the culture medium, the growth regulators (Butiuc-keul et *al*., 2008) and on the genotype and environmental conditions (Visoiu et *al*., 1989). The medium is comprised of basal salts and essential nutrients that plant requires for proper growth and development (Wong, 2009). However, Murashige and Skoog (1962) medium was judged to be the best overall choice due to its common usage in industry. Wong (2009) found that axillary bud explants cultured on MS medium proliferated better than shoot tip explants. While, Sajid and Ahmed (2008) found that 75% concentrations of MS macronutrients worked best for the genotypes "019972" and "Sunder Khani". Jia et *al*.,(1992) stated that half strength MS medium with 1.0 mgl^{-1} BA+0.2 mgl^{-1} indole acetic acid (IAA) promoted the formation and development of grape shoots.

The obvious known fact that cytokinins promote cell division and cell expansion in plant tissue culture, as a result shoot multiplication occurs. However, different concentrations are often required to yield the optimum shoot proliferation. Many studies have clearly shown that Benzyladenine (BA) is the most effective among other cytokinins for inducing shoot development in Vitis. Chee and Pool (1985) defined the optimum concentration of BAP for shoot multiplication (5.0 µM), for production of larger shoots (2.5µM) and for a maximum expanded nodes on the explant (1.0 µM). Pathirana and McKenzie (2007) mentioned that rapid multiplication of shoot can be achieved by supplementing the MS medium with 0.5 mgl^{-1} BAP. On the other hand, many studies reported that mixtures between cytokinin and auxin gave better multiplication rate than using cytokinin alone. Gok et *al*., (1997) found that the optimum concentrations of the growth regulators for shoot multiplication were 1.0 mgl^{-1} BAP+(0.5 or 1.0) mgl^{-1} indol-3-butyric acid (IBA) for "Perlette", "Italian

Riesling" and "du Lot", and 2.0 mgl^{-1} BAP+(0.5 or 1.0) mgl^{-1} IBA for "Salt Creek".

Li and Eaton (1984) reported that rooting of "Marechal Foch" grape in half strength MS salts was superior to rooting in full strength MS medium. Akbas et *al*., (2004) obtained rooting of grape cv. "Perle ad Csaba" on half strength MS medium with 1.0 mgl^{-1} naphthalene acetic acid (NAA), while Banilas and Korkas (2007) performed rooting of grape on half strength MS medium with IBA at 2.5 or 5.0 µM.

In vitro propagation of grapes was obtained by some authors. Nevertheless, "Black Matrouh" cultivar is endangered and there are only a few individual trees cultivated in some private farms in Egypt so, the propagation through tissue culture is the optimal method of propagation where no adequate amounts of the mother plant for propagation by conventional methods.

Thus, the main objective of this study was to obtain an efficient method for large scale in vitro micropropagation of "Black Matrouh" grape (*Vitis vinifera* L.) via shoot tip and nodal cutting explants by investigating different medium constituents and growth regulators through micropropagation stages.

Material and Methods

This study was carried out during the period 2011 - 2013 at the laboratory of Tissue Culture in the National Gene Bank and Genetic Resources, Giza, Egypt.

Preparation and Sterilization of Plant Material: Apical parts of shoots (5-10 cm long), from local grape "Black Matrouh", were taken from two year-old potted plants grown in the greenhouse at the National Gene Bank and Genetic Resources. After removing leaves, shoots were rinsed thoroughly under running tap water for about 1h, then surface sterilized under laminar flow hood cabinet with 70% ethanol for 1 min followed by sodium hypochlorite solution which was prepared using commercial bleach "Clorox" (5.25% available sodium hypochlorite) at 10% concentration for 10 min with a drop of Tween 20, then followed by three rinses; five minutes for each. At last, sterilized shoots were cut into shoot tip (0.8 cm) and nodal cutting (1.0 cm) and used as explant materials.

Culture Media and Conditions: Medium salt formulate tested were the basal medium of Murashige and Skoog (1962) at full, three-quarter or half strength supplemented with 3.0% sucrose and 0.7% agar. Also, different growth regulators; 6-benzylamino purine (BAP) as a cytokinins, indol-3-butyric acid (IBA) and naphthalene acetic acid (NAA) as auxins were used at different concentrations (mgl^{-1}) in order to choose the optimum concentration for each stage of plant tissue culture. All cultures were kept in growth room at 25±2°C and under photoperiods of 16h day supplied by fluorescent lamp (four lamps per shelf) to provide light intensity of 3000 lux at explants level (30 cm from light).

Initiation Stage: Sterilized explants (shoot tips (0.8 cm) and nodal cuttings (1.0 cm) were cultured in establishment medium which consists of full, three-quarter or half strength MS medium supplemented with BAP at 0.0, 0.2, 0.5, 1.0 or 1.5 mgl^{-1}. Survival percentage (%), number of shoots/explant, and shoot length (cm) were determined after 4 week on establishment medium.

- **Multiplication Stage:** Established shoots developed from establishment stage were divided and subcultured into multiplication medium which consists of either full or three-quarter strength MS medium and supplemented with BAP at 0.00, 0.50, 0.75 or 1.00 mgl^{-1} alone or in combination with IBA at 0.00, 0.10, 0.30 or 0.50 mgl^{-1}. Regeneration percentage (%), number of shoots/explant and shoot length (cm) were determined as means after three subcultures on corresponding multiplication medium (4 weeks for each).
- **Rooting Stage:** To optimize the production of adventitious roots in this stage, one subculture to a growth regulator-free medium for 4 weeks was conducted to reduce the level of cytokinin within the explants, then uniform proliferated microshoots of about 1.5 cm in length were transferred to rooting medium which consists of half strength MS medium and supplemented with either IBA or NAA at 0.0, 0.1, 0.3, 0.5 or 1.0 mgl^{-1}. Rooting percentage (%), number of roots/shoot explant, and the length (cm) of the longest root emerging from the base of each rooted explant were determined after 5 weeks on rooting medium.
- **Hardening Stage:** Plantlets with fully expanded leaves and well developed root system were gently washed with sterile distilled water to remove the adhering gelled medium and put in a fungicide (1.0 gl^{-1}) two min before transplanting

into plastic pots (10x15 cm) filled with sterilized mixture of peatmoss: sand (2:1 by volume) and covered with clear plastic pages to maintain high humidity and then maintained in growth chamber (25±2ºC). Air circulation was gradually increased after plastic bags were cut increasingly holes before being completely removed after about 4 weeks and the plants were ready for greenhouse transplantation with artificial lightening and 80% relative humidity for 8 weeks after which success percent was determined.

- **Statistical Analysis:** The experiments were arranged as factorial experiment in completely randomized design with three replicates; six explants for each replicate. Effect of treatments was tested by ANOVA with least significant difference (LSD) calculated at 0.05 level of significance using Waller and Duncan (1969). Collected data were statistically analyzed using IBM SPSS 22.0 (Software Package for Statistics and Simulation, 2013).

Results and Discussion

- **Initiation Stage:**

The selection of the suitable explants is a crucial step for efficient initiation of micropropagation protocol for grape as well as other woody species (Grenan, 1992). In this study, shoot tips and nodal cuttings were selected as explants. Presumably, apical meristems contain less endophytic contamination and are most vigorous during such rapid growth (Gray and Benton, 1991). Results in Table (1) revealed that three-quarter strength MS medium enhanced significantly the survival percentage compared to full and half strength MS medium. With regard to the effect of BAP concentrations, 0.5 mgl-1 BAP was more effective for establishing of shoot tip explants as it stimulated survival percentage without significant differences compared to 0.2 or 1.0 mgl^{-1} BAP. All shoot tip explants cultured on three-quarter strength MS medium with 0.5 mgl^{-1} BAP were able to establish (100%) without significant difference compared to survival percentage (83.33%) of shoot tip explants cultured on full strength MS medium with 1.0 mgl-1 BAP. Similarly, Sajid and Ahmed (2008) found that MS medium at 75% levels of macronutrients were best for grape genotypes “Sunder Khani” and “019972 accession”. Also, Abido et al. (2013) recorded 100% survival percentage of shoot tip and stem segment explants of grape cv. “Muscat of Alexandria” on MS medium with 0.5 mgl^{-1} BAP.

Table 1. Effect of different MS medium strengths and BAP concentrations on survival percentage of “Black Matrouh” shoot tip explants on establishment medium.

BAP (mgl^{-1})	MS medium strength			Mean
	Full	Three-quarter	Half	
BAP-free	44.44^{d}	77.78^{b}	50.00^{cd}	**57.40^{B}**
0.2	66.67^{bc}	77.78^{b}	66.67^{bc}	**70.37^{AB}**
0.5	66.67^{bc}	100.00^{a}	77.78^{b}	**81.48^{A}**
1.0	83.33^{ab}	66.67^{bc}	66.67^{bc}	**72.22^{AB}**
1.5	50.00^{cd}	66.67^{bc}	44.44^{d}	**53.70^{B}**
Mean	**62.22^{B}**	**77.78^{A}**	**61.11^{B}**	

Means followed by a letter in common in the same column are not significantly different at 0.05 level of probability.

Results in Table (2) and Fig. (1A) indicated that full strength MS medium improved significantly the number of shoots/explant compared to three-quarter and half strength MS medium. Meanwhile, adding BAP at 0.5 or 1.0 mgl^{-1} to establishment medium increased significantly the number of shoots/explant, while BAP-free medium give rise to only one shoot. The highest number of shoots/explant (1.86) was obtained on full strength MS medium with 0.5 mgl^{-1} BAP. However, this finding could be attributed to the mode of action of BAP at these concentrations (0.5-1.0 mgl-1) on stimulation both cell division and promote growth of axillary shoots in plant tissue culture as reported by George et *al*.,(2008). In this line, El-Din et *al*., (1997) found that the highest shoot number/apical meristem explant was recorded for cv.”Fry” on MS medium with 0.5 mgl-1 BA. On contrast, Aazami (2010) reported that MS medium with 1.5 mgl^{-1} BA or with 1.5 mgl^{-1} BA+1.0 mgl^{-1} IBA produced the highest average number of shoots/cultured apex in both grape cultivars “Soltanin” and “Sahebi”.

Table 2. Effect of different MS medium strengths and BAP concentrations on number of shoots/ shoot tip explant of "Black Matrouh" on establishment medium.

BAP (mgl^{-1})	MS medium strength			Mean
	Full	Three-quarter	Half	
BAP-free	1.00^{e}	1.00^{e}	1.00^{e}	**1.00^{C}**
0.2	1.44^{bc}	1.17^{de}	1.06^{e}	**1.22^{B}**
0.5	1.86^{a}	1.17^{de}	1.11^{de}	**1.44^{A}**
1.0	1.50^{b}	1.50^{b}	1.28^{cd}	**1.43^{A}**
1.5	1.00^{e}	1.00^{e}	1.06^{e}	**1.02^{C}**
Mean	**1.40^{A}**	**1.17^{B}**	**1.10^{B}**	

Means followed by a letter in common in the same column are not significantly different at 0.05 level of probability.

Results in Table (3) demonstrated that the highest shoot length was observed on three-quarter strength MS medium without significant difference compared to full strength MS medium. Nevertheless, shoot length of shoot tip explants dose not submit to the same principle of shoot number; i.e. values of shoot length appeared to decrease gradually with increasing BAP concentrations from 0.2, 0.5, 1.0 to 1.5 mgl^{-1}, while the highest shoot length was noticed on BAP-free medium. The highest shoot length (2.14 cm) was recorded on BAP-free three-quarter strength MS medium without significant difference compared to shoot length (2.12 cm) of explants cultured on three-quarter strength MS medium with 0.2 mgl^{-1} BAP. While the shortest shoots (1.24 cm) were noticed on half strength MS medium with 1.0 mgl^{-1} BAP and the reason may be due to the antagonism relation between high number of shoots and their length and the role of adverse effect of cytokinin on cell elongation (George et *al*., 2008). This is in accordance with Abido et *al*., (2013) who mentioned that the absence of BAP from culture medium gave rise to the longest shoot obtained from shoot tip explants of grape "Muscat of Alexandria".

Table 3. Effect of different MS medium strengths and BAP concentrations on shoot length (cm) "Black Matrouh" explants on establishment medium.

BAP (mgl^{-1})	MS medium strength			Mean
	Full	Three-quarter	Half	
BAP-free	1.73^{b}	2.14^{a}	1.69^{bc}	**1.86^{A}**
0.2	1.81^{b}	2.12^{a}	1.35^{e}	**1.76^{AB}**
0.5	1.82^{b}	1.60^{bcd}	1.49^{cde}	**1.64^{BC}**
1.0	1.69^{bc}	1.78^{b}	1.24^{e}	**1.57^{BC}**
1.5	1.43^{de}	1.59^{bcd}	1.39^{de}	**1.47^{C}**
Mean	**1.70^{A}**	**1.85^{A}**	**1.43^{B}**	

Means followed by a letter in common in the same column are not significantly different at 0.05 level of probability.

Other investigations had proved that propagation by axillary shooting was the most applicable and reliable method for in vitro propagation of grapes (Banilas and Korkas, 2007). Results in Table (4) illustrated that three-quarter strength MS medium enhanced significantly the survival percentage compared to half strength MS medium. While, adding BAP at 0.2, 0.5 or 1.0 mgl^{-1} to establishment medium did not improve significantly the survival percentage compared to BAP-free medium or medium with BAP at 1.5 mgl^{-1}. The lowest survival percentage (50.00%) was noticed on half strength MS medium with 1.5 mgl^{-1} BAP. On contrast, Mukherjee et *al*., (2010) found that half strength MS medium with 2.0 mgl^{-1} BAP was optimum for establishment of nodal explants of grape "de Grasset".

Table 4. Effect of different MS medium strengths and BAP concentrations on survival percentage of "Black Matrouh" nodal explants on establishment medium.

BAP (mgl^{-1})	MS medium strength			Mean
	Full	Three-quarter	Half	
BAP-free	77.78^{ab}	72.22^{ab}	61.11^{bc}	**70.37^{A}**
0.2	83.33^{ab}	83.33^{a}	66.67^{abc}	**77.78^{A}**
0.5	72.22^{ab}	88.89^{a}	88.89^{a}	**83.33^{A}**
1.0	77.78^{ab}	83.33^{a}	77.78^{ab}	**79.63^{A}**
1.5	77.78^{ab}	83.33^{a}	50.00^{c}	**70.37^{A}**
Mean	**77.78^{AB}**	**82.22^{A}**	**68.89^{B}**	

Means followed by a letter in common in the same column are not significantly different at 0.05 level of probability.

With respect to the number of shoots formed per nodal explant, results in Table (5) and Fig. (1B) indicated that full strength MS medium stimulated shoot formation compared to half strength MS medium. In addition, the number of shoots formed per nodal explant was significantly affected by the present of BAP at 1.0 or 1.5 mgl^{-1}. On the other hand, only the main shoot developed while the growth of axillary buds was inhibited on BAP-free medium and medium with BAP at 0.2 mgl^{-1}. In this line, Han et *al*., (2003) observed a tendency for more than one bud to initiate growth on BAP containing media. They also demonstrated that the limited degree of shoot micropropagation in the growth regulator-free medium may be due to the presence of small endogenous cytokinin content in the explants. The highest number of shoots/explant (1.67) was obtained on full strength MS medium with 1.0 mgl^{-1} BAP. This finding could be attributed to the mode of action of BAP at 1.0 mgl^{-1} on stimulating both cell division and promote of axillary shoots in pant tissue culture as reported by George et *al*., (2008). These results agree with those obtained by Shim et *al*., (2001) who induced multiple shoots from *Vitis* hybrid "Campbell Early" in a medium containing 1.0 mgl^{-1} BA. Also, Jaskani et al. (2008) obtained the best shoot formation from nodal segments on MS medium with 5.0 µM BA.

Table 5. Effect of different MS medium strengths and BAP concentrations on number of shoots/ nodal explantof "Black Matrouh" on establishment medium.

BAP (mgl^{-1})	MS medium strength			Mean
	Full	Three-quarter	Half	
BAP-free	1.00^{f}	1.00^{f}	1.00^{f}	**1.00^{C}**
0.2	1.00^{f}	1.00^{f}	1.00^{f}	**1.00^{C}**
0.5	1.11^{ef}	1.22^{cde}	1.17^{de}	**1.17^{B}**
1.0	1.67^{a}	1.22^{cde}	1.28^{cd}	**1.39^{A}**
1.5	1.50^{b}	1.38^{bc}	1.22^{cde}	**1.36^{A}**
Mean	**1.26^{A}**	**1.16^{AB}**	**1.13^{B}**	

Means followed by a letter in common in the same column are not significantly different at 0.05 level of probability.

Concerning the shoot length, results in Table (6) cleared that full strength MS medium showed the highest shoot length with significant differences compared to three-quarter or half strength MS medium. Meanwhile, shoot elongation was not affected significantly by the increased BAP concentration in the establishment medium. The highest shoot length (2.03 cm) was noticed on full strength MS medium with 0.5 mgl^{-1} BAP without significant difference compared to shoot length (1.94 cm) of explants cultured on full strength MS medium with 1.0 mgl^{-1} BAP. In this concern, Sajid and Ahmed (2008) found that maximum shoot length was recorded on MS medium containing 100% levels of macronutrients and micronutrients, whereas moderate shoot lengths were recorded on MS medium containing either 75% or 50% levels of each of the macronutrients and micronutrients. Abido et al. (2013) mentioned that the highest mean shoot length per stem segment explant of grape "Muscat of Alexandria" was obtained on MS medium supplemented with 1.0 mgl^{-1} BAP.

Table 6. Effect of different MS medium strengths and BAP concentrations on shoot length (cm) "Black Matrouh" explants on establishment medium.

BAP (mgl^{-1})	MS medium strength			Mean
	Full	Three-quarter	Half	
BAP-free	1.44^{d}	1.56^{cd}	1.42^{d}	**1.47^{A}**
0.2	1.76^{bc}	1.39^{d}	1.50^{cd}	**1.55^{A}**
0.5	2.03^{a}	1.44^{d}	1.40^{d}	**1.62^{A}**
1.0	1.94^{ab}	1.56^{cd}	1.48^{d}	**1.66^{A}**
1.5	1.46^{d}	1.42^{d}	1.47^{d}	**1.45^{A}**
Mean	**1.73^{A}**	**1.47^{B}**	**1.45^{B}**	

Means followed by a letter in common in the same column are not significantly different at 0.05 level of probability.

- **Multiplication Stage:**

Results in Table (7) indicated that established explants cultured on either full or three-quarter strength MS medium showed high ability to regenerate new shoots without significant difference between them. Meanwhile, adding growth regulators (cytokinin and auxin) to multiplication medium enhanced significantly the regeneration percentage compared to growth regulator-free medium. These results agree with those of Tao et *al*., (2005) who recorded the highest regeneration percentage of adventitious buds of grape on MS medium with (1.0 BA+0.01 IBA) mgl^{-1}.In this respect, Nike et *al*., (1999) declared that cytokinins are effective for multiplication rate when used in combination with auxin at appropriate balance. However, insufficient concentrations cannot induce shoot proliferation; also excessive levels of cytokinin in medium can inhibit shoot multiplication and however, can elicit vitrification appearance (Van Staden *et a*l., 2008). While, Torrey and Reinert (1961) found that auxin working to increase the activating enzymes that break down starch and has the ability to move the active leading to increased multiplication of organogenesis. Auxins are capable to control various distinctive processes such as promotion of stem elongation and growth and they are not affective against shoot proliferation.

It was obvious from the results in Table (8) and Fig. (1C) that multiplication medium which consists of three-quarter strength MS medium was more effective on stimulating number of proliferated shoots/explants compared to full strength MS medium (2.52 and 2.06, respectively). Also, shoot multiplication was totally absent at growth regulator-free medium giving only one branched shoot; while the highest number of shoots/explant (3.67) was obtained on three-quarter strength MS medium supplemented with (0.75 BAP+ 0.50 IBA) mgl^{-1} without significant difference compared to number of shoots (3.52) proliferated from explants cultured on three-quarter strength MS medium supplemented with (1.00 BAP+0.10 IBA) mgl^{-1}. These results agree with those obtained by Sajid et *al*., (2006) who found that grape explants grown on media, which were either devoid of any growth regulator or containing low concentrations of BAP or auxins, showed shoot elongation only without shoot multiplication in numbers. However, a maximum number of shoots was harvested from the medium containing 0.75 mgl^{-1} BAP. On the other hand, previous studies reported that mixtures between cytokinin and auxin gave better multiplication rate than using cytokinin alone. Gok et al. (1997) mentioned that the optimum concentrations of the growth regulators for shoot multiplication were 1.0 mgl^{-1} BAP + (0.5 or 1.0) mgl^{-1} IBA for "Perlette", "Italian Riesling" and "du Lot". On contrast, Sajid et *al*., (2006) found that adding IAA or NAA to multiplication medium have no effect on shoot multiplication of "Wild Grape" and "Sundar Khani" grapes.

Table 7. Effect of different MS medium strengths and growth regulator treatments on regeneration percentage of "Black Matrouh" after 3 subcultures on multiplication medium (4 weeks for each).

Treatment (mgl^{-1})	MS medium strength		Mean
	Full	Three-quartet	
Growth regulator-free	66.67^{d}	77.78^{c}	$\mathbf{72.22^{D}}$
0.50BAP + 0.00 IBA	88.89^{b}	92.59^{ab}	$\mathbf{90.74^{BC}}$
0.50BAP +0.10 IBA	100.00^{a}	100.00^{a}	$\mathbf{100.00^{A}}$
0.50BAP +0.30 IBA	100.00^{a}	96.30^{ab}	$\mathbf{98.15^{AB}}$
0.50BAP +0.50 IBA	92.59^{ab}	100.00^{a}	$\mathbf{96.30^{ABC}}$
0.75BAP +0.00 IBA	92.59^{ab}	96.30^{ab}	$\mathbf{94.44^{ABC}}$
0.75BAP +0.10 IBA	100.00^{a}	96.30^{ab}	$\mathbf{98.15^{AB}}$
0.75BAP +0.30 IBA	100.00^{a}	100.00^{a}	$\mathbf{100.00^{A}}$
0.75BAP +0.50 IBA	100.00^{a}	100.00^{a}	$\mathbf{100.00^{A}}$
1.00BAP +0.00 IBA	88.89^{b}	88.89^{b}	$\mathbf{88.89^{C}}$
1.00BAP +0.10 IBA	100.00^{a}	100.00^{a}	$\mathbf{100.00^{A}}$
1.00BAP +0.30 IBA	92.59^{ab}	96.30^{ab}	$\mathbf{94.44^{ABC}}$
1.00BAP +0.50 IBA	100.00^{a}	96.30^{ab}	$\mathbf{98.15^{AB}}$
Mean	$\mathbf{94.02^{A}}$	$\mathbf{95.44^{A}}$	

Means followed by a letter in common in the same column are not significantly different at 0.05 level of probability.

Table 8. Effect of different MS medium strengths and growth regulator treatments on number of shoots/explant of "Black Matrouh" after 3 subcultures on multiplication medium (4 weeks for each).

Treatment (mgl^{-1})	MS medium strength		Mean
	Full	Three-quartet	
Growth regulator-free	1.00^{k}	1.00^{k}	$\mathbf{1.00^{F}}$
0.50BAP + 0.00 IBA	1.15^{jk}	1.22^{jk}	$\mathbf{1.19^{EF}}$
0.50BAP +0.10 IBA	1.67^{i}	2.63^{d-g}	$\mathbf{2.15^{D}}$
0.50BAP +0.30 IBA	2.30^{fgh}	2.44^{e-h}	$\mathbf{2.37^{CD}}$
0.50BAP +0.50 IBA	2.11^{h}	2.41^{e-h}	$\mathbf{2.26^{CD}}$
0.75BAP +0.00 IBA	2.26^{gh}	2.96^{cd}	$\mathbf{2.16^{C}}$
0.75BAP +0.10 IBA	2.30^{fgh}	2.89^{cd}	$\mathbf{2.59^{BC}}$
0.75BAP +0.30 IBA	2.41^{e-h}	2.74^{de}	$\mathbf{2.57^{BC}}$
0.75BAP +0.50 IBA	2.67^{def}	3.67^{a}	$\mathbf{3.17^{A}}$
1.00BAP +0.00 IBA	1.48^{ij}	1.41^{ij}	$\mathbf{1.44^{E}}$
1.00BAP +0.10 IBA	2.56^{d-g}	2.78^{cde}	$\mathbf{2.52^{BCD}}$
1.00BAP +0.30 IBA	2.63^{d-g}	3.52^{ab}	$\mathbf{3.07^{A}}$
1.00BAP +0.50 IBA	2.56^{d-g}	3.15^{bc}	$\mathbf{2.85^{AB}}$
Mean	$\mathbf{2.06^{B}}$	$\mathbf{2.52^{A}}$	

Means followed by a letter in common in the same column are not significantly different at 0.05 level of probability.

With regard to shoot length, results in Table (9) revealed that full strength MS medium enhanced significantly shoot elongation compared to three-quarter strength MS medium (1.93 and 1.76 cm, respectively). The highest shoot length (4.94 cm) was observed on full strength growth regulator-free medium, while increasing BAP concentrations had a retarding effect on shoot length. The presence of IBA in multiplication medium did not improve shoot elongation; this may due to the antagonism relation between high number of shoots and their length (Al-Dhaher, 2010). These finding are in accordance with those of Sajid et al. (2006) who found that increasing BAP from 0.00 to 0.75 mgl^{-1} had a retarding effect on shoot length. On contrast, they found that the presence of IAA or NAA in these media reduced the retarding effect of BAP on shoot length.

Table 9. Effect of different MS medium strengths and growth regulator treatments on shoot length (cm) of "Black Matrouh" after 3 subcultures on multiplication medium (4 weeks for each).

Treatment (mgl^{-1})	MS medium strength		Mean
	Full	Three-quartet	
Growth regulator-free	4.94^{a}	3.64^{b}	**4.29^{A}**
0.50BAP +0.00 IBA	1.98^{c}	1.84^{cde}	**1.91^{B}**
0.50BAP +0.10 IBA	1.97^{c}	1.58^{e-h}	**1.77^{BC}**
0.50BAP +0.30 IBA	1.67^{def}	1.93^{cd}	**1.80^{BC}**
0.50BAP +0.50 IBA	1.49^{fgh}	1.82^{cde}	**1.66^{B-E}**
0.75BAP +0.00 IBA	1.99^{c}	1.53^{fgh}	**1.76^{BCD}**
0.75BAP +0.10 IBA	1.58^{e-h}	1.64^{efg}	**1.61^{CDE}**
0.75BAP +0.30 IBA	1.66^{def}	1.45^{fgh}	**1.56^{CDE}**
0.75BAP +0.50 IBA	1.53^{fgh}	1.56 $^{e-h}$	**1.49^{DE}**
1.00BAP +0.00 IBA	1.84^{cde}	1.37^{gh}	**1.60^{CDE}**
1.00BAP +0.10 IBA	1.53^{fgh}	1.35^{h}	**1.44^{E}**
1.00BAP +0.30 IBA	1.45^{fgh}	1.64^{efg}	**1.55^{CDE}**
1.00BAP +0.50 IBA	1.41^{fgh}	1.65^{d-g}	**1.53^{CDE}**
Mean	**1.93^{A}**	**1.76^{B}**	

Means followed by a letter in common in the same column are not significantly different at 0.05 level of probability.

- **Rooting Stage:**

Uniform proliferated microshoots of about 1.5 cm in length formed in the multiplication stage were used for root formation. The influence of various concentrations of IBA or NAA was investigated. From results in Table (10), it could be concluded that rooting percentage of "Black Matrouh" shoots was not affected by the two auxins under investigation, as no significant difference was observed between rooting percentage on medium with IBA or NAA (75.56 and 71.11%, respectively), while rooting percentage increased from 55.56% to 83.33% with increasing auxin concentration from 0.0 to 1.0 mgl^{-1}. Rooting percentage was highest when shoots were cultured on half strength MS medium with 1.0 mgl^{-1} IBA (88.89%) and with 0.5 mgl^{-1} IBA or NAA (83.33%) without significant differences compared to rooting medium with 0.1 mgl^{-1} IBA or 1.0 mgl^{-1} NAA (77.78%) and rooting medium with 0.3 mgl^{-1} IBA or NAA (72.22%). Shoot explants cultured on auxin-free half strength MS medium showed low rooting efficiency as they exhibited the lowest rooting percentage (55.56%). In micropropagation, auxins are known for their ability to promote adventitious root formation (Adams and Early, 2004). In this concern, Singh and Brar (1993) mentioned that more than 85% of adventitious shoots of grape cvs. "Thompson Seedless" and "Perlette" rooted successfully on MS medium containing IBA at 1.0 mgl^{-1}. Moreover, Aazami (2010) performed rooting of grape cultivars "Soltanin" and "Sahebi" on MS medium containing 0.5 mgl^{-1} NAA. While Diab et *al*., (2011) demonstrated that NAA was not suitable for rooting as the percentage of rooting was 35% only.

Table 10. Effect of different auxin concentrations on rooting percentage of "Black Matrouh" shoot explants after 5 weeks on rooting medium.

Concentration (mgl^{-1})	Auxin		Mean
	IBA	NAA	
Auxin-free	55.56^{c}	55.56^{c}	**55.56^{B}**
0.1	77.78^{ab}	66.67^{bc}	**72.22^{AB}**
0.3	72.22^{abc}	72.22^{abc}	**72.22^{AB}**
0.5	83.33^{ab}	83.33^{ab}	**83.33^{A}**
1.0	88.89^{a}	77.78^{ab}	**83.33^{A}**
Mean	**75.56^{A}**	**71.11^{A}**	

Means followed by a letter in common in the same column are not significantly different at 0.05 level of probability.

With regard to the number of roots formed per shoot, results in Table (11) and Fig. (1D) demonstrated that NAA proved to be better in stimulating root formation compared to IBA (4.47 and 3.56 roots/shoot, respectively). In addition, shoots rooted in medium with auxin (NAA or IBA) produced more roots than those rooted in medium without auxin. The highest number of roots/shoot (5.50) was observed on rooting medium with 0.5 mgl^{-1} NAA without significant differences compared to number of roots produced from shoots on medium with 0.3 or 1.0 mgl^{-1} NAA (4.94 and 4.50 roots/shoot, respectively). There is substantial evidence that auxins play a crucial role in adventitious root formation and all auxins are rapidly metabolized after uptake but there are differences in their effectiveness (Van der krieken et *al*., 1992). Chhun et al. (2004) mentioned that responses can vary depending on the auxin application mode and concentration as well as on the tissue's sensitivity to exogenous auxin. In this respect, Gray and Benton (1991) mentioned that auxin apparently accelerated all aspects of in vitro rooting. Banilas and Korkas (2007) indicated that the addition of IBA, particularly at 5.0 µM, facilitated rooting percentage and the number of roots/shoot.

Table 11. Effect of different auxin concentrations on number of roots/shoot explant of "Black Matrouh" after 5 weeks on rooting medium.

Concentration (mgl^{-1})	Auxin		Mean
	IBA	NAA	
Auxin-free	3.11^{e}	3.11^{e}	**3.11^{B}**
0.1	3.33^{de}	4.28^{bcd}	**3.81^{AB}**
0.3	3.83^{cde}	4.94^{ab}	**4.39^{A}**
0.5	4.06^{b-e}	5.50^{a}	**4.78^{A}**
1.0	3.44^{de}	4.50^{abc}	**3.97^{A}**
Mean	**3.56^{B}**	**4.47^{A}**	

Means followed by a letter in common in the same column are not significantly different at 0.05 level of probability.

As for root length, results in Table (12) revealed that root elongation was not affected by the two auxins under investigation. No significant difference was observed between root length on medium with IBA or NAA (4.08 and 3.79 cm, respectively). Also, adding different concentrations of auxin to rooting medium enhanced root length compared to free-auxin medium which exhibited the shortest roots (3.25 cm). The highest root length (5.06 cm) was observed after 5 weeks on rooting medium with 0.5 mgl^{-1} IBA without significant differences compared to root length (4.11 and 4.19 cm) of shoots on medium with 0.1 and 0.3 mgl^{-1} IBA respectively, and root length (4.17 and 4.11 cm) of shoots on medium with 0.3 and 0.5 mgl^{-1} NAA respectively.

Table 11. Effect of different auxin concentrations on number of roots/shoot explant of "Black Matrouh" after 5 weeks on rooting medium.

Concentration (mgl^{-1})	Auxin		Mean
	IBA	NAA	
Auxin-free	3.25^{b}	3.25^{b}	**3.25^{B}**
0.1	4.11^{ab}	3.58^{b}	**3.85^{AB}**
0.3	4.19^{ab}	4.17^{ab}	**4.18^{A}**
0.5	5.06^{a}	4.11^{ab}	**4.59^{A}**
1.0	3.81^{b}	3.83^{b}	**3.82^{AB}**
Mean	**4.08^{A}**	**3.79^{A}**	

Means followed by a letter in common in the same column are not significantly different at 0.05 level of probability.

- **Hardening Stage:**

One of the most important steps in tissue culture is the hardening of plantlets to free living conditions. This stage involves transfer of plantlets from aseptic condition to green house and ultimately to the final location (environment). A successful tissue culture method of propagation must result in re-establishment in soil of high frequency of the tissue culture derived plant.

After two months of acclimatization, survival percentage of rooted plantlets developed on half strength MS rooting medium reached up to 60.00 % for "Black Matrouh" when transferred and planted on plastic pots filled with a sterilized mixture of peatmoss and sand (2:1). Plants were approximately 10 cm tall and were ready to be transferred to bigger pots for further growth (Fig. 1E). However, acclimatization rate could be improved considerably so that these acclimated plantlets could be transplanted to the field. In this concern, Abido *et al*. (2013) clarified that leaving the plantlets that have roots in the rooting medium for longer period of time increased the efficiency of roots which led to increase the survival percentages of acclimatized plantlets of grape cv. "Muscat of Alexandria".

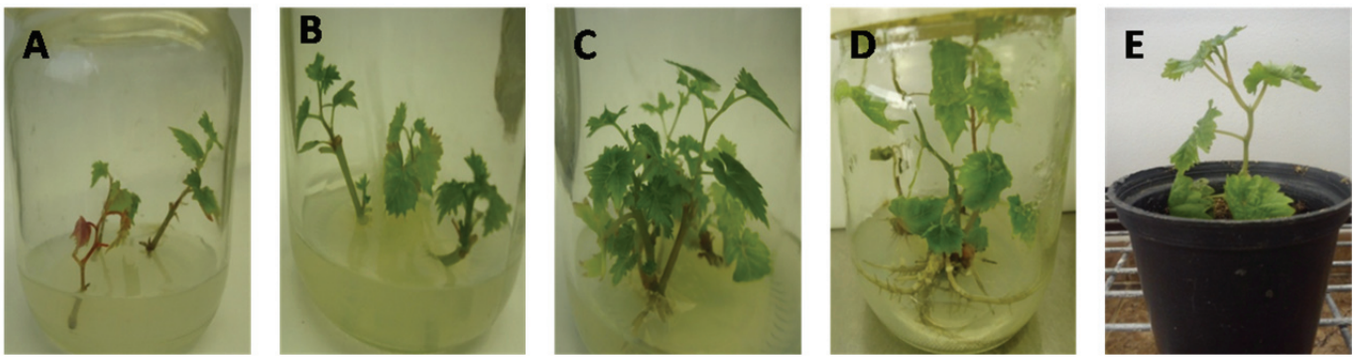

Fig.1. Micropropagation stages of *Vitis vinifera* L. cv. "Black Matrouh"

A. Shoot tip establishment	B. Nodal cutting establishment
C. Multiple shoot proliferation	D. *In vitro* rooted plantlets after 5 weeks of culture
E. Plantlets acclimatized in greenhouse.	

Conclusions

The technique developed and described here for *in vitro* micropropagation of grape (*Vitis vinifera* L.) cv. "Black Matrouh" can be effectively used for germplasm conservation for protecting the grape biodiversity available in the country from severe losses arising from epidemics. Besides facilitating the germplasm exchange, the technique by virtue of maximizing shoot mass and subsequent root induction in vitro ensure that the plants are produced on large scale without losses and in a minimum possible time.

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