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Effects of cytokinins and auxins on the micropropagation of a local grapevine (*Vitis vinifera* L.) cultivar, using nodal explants

Osman A. Ali[1], Mohamed A. Ali[2] and Abdel Moneim M. Salama[1]

[1]Faculty of Agriculture and Natural Resources, University of Gezira, Wad Medani, Sudan.
[2]Agricultural Research Corporation, Wad Medani, Sudan.

ABSTRACT

This study was conducted in the Plant Tissue Culture Unit of the Agricultural Research Corporation, Wad Medani, Sudan, during the years 2006- 2007. The objectives were to develop an *in vitro* technique for the propagation of a local cultivar of grapevine. The basal Murashige and Skoog medium was supplemented with benzylaminopurine (BAP) and kinetin both at concentrations of 0.0, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 mg/l. Isopentenyladenine (2iP) and BAP were also used at concentrations of 0.0, 1.0, 1.5, and 2.0 mg/l. The interaction effects of indole acetic acid (IAA) at concentrations of 0.0, 0.25, 0.5 and 1.0 mg/l with BAP at 1.0 or 2.0 mg/l and indole butyric acid (IBA) at concentrations of 0.0, 0.1, 0.3, and 0.5 mg/l with 2iP at 1.0 or 2.0 mg/l were also tested. For *in vitro* rooting, plantlets were cultured on MS medium supplemented with different concentrations of NAA (0.0, 0.25, 0.5, and 1.0 mg/l). The best morphogenesis of shoots was achieved on MS medium supplemented with BAP at 1.0-1.5 mg/l. The morphogenetic response increased with time and the best response was attained after six weeks. Higher concentrations of BAP (2.0-8.0 mg/l) resulted in abnormal growth of plantlets. The combination of BAP with IAA did not improve shoot morphogenesis. The morphogenesis of the nodal explants was similar on kinetin and 2iP which resulted in a single shoot per explant. The combinations of 2iP at different concentrations with IBA did not improve shoot morphogenesis compared to 2iP alone. The best rooting of regenerated shoots of grapevine was achieved on MS medium supplemented with 0.25 and 0.5 mg/l NAA after four weeks.

INTRODUCTION

Grapes (*Vitis vinifera* L.) are adapted to a wide range of climates. The best production occurs in the temperate regions but some cultivars tolerant to high temperatures have been introduced to tropical and subtropical countries (Shoemaker, 1978). In the Sudan, grapes are grown on a very small scale in nurseries as ornamental plants, because it grows as an evergreen climbing vine, and usually used for arbors, arcade or on pergola and fence. Recently, in northern Omdurman along the Nile, grapes are grown commercially in an area of 30-40 ha (Osman *et al.,* 2009). Lack of cultivars tolerant to high temperature and little knowledge about the training technique may be the limiting factors to grape production in the Sudan. It is possible to overcome these by introducing the proper cultivars that can tolerate high temperature and do well in tropical regions and by training personnel.

Grapes, like many fruit trees, can be propagated asexually. Most grapevines are propagated through traditional techniques by hard wood cuttings, layering of canes or by grafting. This could be difficult because rooting of cuttings is usually poor (Avery, 1999). Layering technique is used primarily for replacing missing vines in established vineyards. It is cumbersome for the production of large numbers of plants. Grafting technique is a tedious method used widely in many infested area with phylloxera and nematodes. Propagation of grapes in the Sudan by traditional techniques faced some problems, and did not provide enough plants to meet growers needs.

Micropropagation has been a well-established methodology for *in vitro* regeneration of grapevine through shoot apical meristem culture or adventitious bud formation (Banilas and Korkas, 2007). Micropropagation has a number of advantages over traditional plant propagation techniques. It is a rapid multiplication method of stock plant material and also used to produce disease-free plants.

The objectives of the present study were to investigate the optimal levels of cytokinins and auxin concentrations for the induction of shoots and roots of a local cultivar of grape (*Vitis vinifera* L.) by using nodal explants.

MATERIALS AND METHODS

The study was conducted at the laboratory of Plant Tissue Culture Research Unit of Agricultural Research Corporation, Wad Medani, Sudan, during the period 2006 to 2007. Cuttings from new growing shoots were collected from a local cultivar of grapevine (*Vitis vinifera* L.) developed and maintained in the greenhouse of the Tissue Culture Research Unit. Explants were sterilized by rinsing twice in distilled water for five minutes, dipping in 70% ethanol for 10 seconds, followed by washing with distilled water and shaking in 20% commercial bleach (Clorox) for 20 minutes. Two drops of Tween 20 were added to the solution of Clorox as a wetting agent, then washed three times with distilled water. Single explants with prominent eyes were excised to one cm long and placed into test tubes containing Murashige and Skoog (1962) medium supplemented with different concentrations of BAP or kinetin at 0.0, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 mg/l. Explants were also cultured on MS medium supplemented with different concentrations of 2iP or BAP at 0.0, 1.0, 1.5, and 2.0 mg/l. Combination of BAP or 2iP with IBA or IAA with different concentrations were also tested. For *in vitro* rooting of local grapevine both test tubes and large vessels were used. Plantlets were cultured on MS medium supplemented with different concentrations of NAA (0.0, 0.25, 0.5, and 1.0 mg/l). The cultures were incubated at 25°C with 16 hrs of light. All experiments were conducted in aseptic conditions.

Experiments were arranged in a completely randomized design (CRD), with five replicates. Data on percentage of explants with shoot, number of shoots per explant, number of nodes per main shoot, and length of primary shoot (cm) were recorded after six weeks of incubation. Data on percentage of plants with roots and number of roots per explant were recorded after four weeks of incubation. Statistical analysis of the data was conducted using Mstat-C package and mean separation was done using the least significant difference (LSD) test.

RESULTS AND DISCUSSION

Morphogenesis of nodal explants of grapevine on MS medium supplemented with different concentrations of BAP

The addition of cytokinins to the culture medium was essential for bud sprouting and *in vitro* multiplication of shoots. The percentages of explants producing shoots were not significantly different between the different concentrations of BAP (Table1). However, the number of regenerated shoots per explant increased significantly with BAP concentration up to 1.0 mg/l and then declined at higher concentrations. Higher concentrations of BAP (2.0-8.0 mg/l) resulted in abnormal growth of plantlets, short or swollen shoots with rosette or unexpanded leaves (Plate 1). These results are in line with those reported by Bigger and Reed (2004) and Ibanez *et al.* (2005) who reported that high concentrations of cytokinin (8 µM BA) produced plantlets of lower quality than lower concentrations (2 and 4 µM BA).

Table 1. Effect of different concentrations of BAP on percentage of explants with shoot, number of shoots per explant, number of nodes and height of main shoot of grapevine on MS medium after six weeks.

BAP (mg/l)	Explants with shoots(%)	No.of shoots per explant	No. of nodes per main shoot	Length of main shoot (cm)
0.0	100	1.0 b	1.5 c	1.9 abc
0.25	100	1.1 b	2.2 b	2.2 ab
0.5	100	1.5 ab	1.8 bc	2.1 abc
1.0	100	2.2 a	3.0 a	2.4 a
2.0	100	1.2 b	1.4 c	1.1 d
4.0	100	1.4 ab	1.2 c	1.3 cd
6.0	100	1.0 b	1.2 c	0.8 d
8.0	100	1.3 b	1.4 c	1.5 bcd
Mean	100	1.3	1.7	1.7
SE	0.0	0.3	0.2	0.3
Sign.	NS	*	***	***

Means in columns followed by the same letter(s) are not significantly different at 5% level of probability according to the least significant difference test (LSD).
Significant at * = P≤ 0.05. Very highly significant *** =P≤ 0.001. NS = not significant.

Plate 1. Morphogenesis of nodal explants of grapevine on MS medium supplemented with different concentrations of BAP, after six weeks.

The number of nodes per main shoot was significantly higher on MS medium supplemented with 1.0 mg/l BAP compared to other concentrations. The main shoots were significantly taller at lower concentrations of BAP (up to 1.0 mg/l) compared to higher concentrations. The number of nodes increased with shoot length at relatively low concentrations of BAP (up to 1.0 mg/l), whereas, at higher concentrations (2.0 up to 8.0 mg/l) the number of nodes per shoot and the height of the main shoot were reduced (Table 1).

According to the results obtained, which showed normal growth at lower concentrations of BAP, concentrations of 0.0, 1.0, 1.5, and 2.0 mg/l were used (Table 2). The percentages of explants with shoots were not significantly different between the concentrations of BAP (0.0-2.0mg/l). Significantly higher number of shoots per explants were formed on MS medium supplemented with 1.0, 1.5 and 2.0 mg/l which induced three shoots on average after six weeks compared with MS medium devoid of BAP (Plate 2). Similar results were obtained by Banilas and Korkas (2007), who found a single shoot induced on nodal buds of grapevine on MS medium lacking growth regulators.

Table 2. Effect of different concentrations of BAP on percentage of explants with shoot, number of shoots per explant, number of nodes and height of main shoot of grapevine cultured on MS medium, after six weeks.

BAP(mg/l)	Explants with shoots (%)	No. of shoots per explant	No. of nodes per main shoot	Height of shoot (cm)
0.0	100	1.0 b	3.0 a	2.9 b
1.0	100	2.7 a	3.4 a	4.1 a
1.5	100	3.4 a	3.1 a	2.7 b
2.0	100	3.5 a	2.2 b	2.4 b
SE(±)	0.0	0.3	0.2	0.3
Sig. level	NS	***	**	***

Means in columns followed by the same letter(s) are not significantly different at 5% level of probability according to the least significant difference test (LSD).
Highly significant at. * * =P≤ 0.01. Very high significant ***=P≤0.001. NS = not significant

Plate 2. Effect of different concentrations of BAP on number of shoots regenerated from nodal explants of grapevine cultured on MS medium, after six weeks.
(BAP: 1= 0.0 mg/l., 2=1.0 mg/l., 3=1.5 mg/l. and 4=2.0 mg/l)

Higher numbers of nodes were induced on MS medium lacking BAP and on MS supplemented with 1.0 and 1.5 mg/l BAP compared to 2.0 mg/l (Table 2). Height of plantlets increased significantly on MS medium supplemented with 1.0 mg/l compared with other concentrations, which induced comparable plant heights (Table2). These results are in general accordance with those reported by Muhammad *et al*. (2008), Banilas and Korkas (2007) and Mhatre *et al*. (2000), who found that the optimum rate of shoot multiplication induced by BAP ranged from 5.0 to 10.0 µM.

Effect of BAP combined with IAA on morphogenesis of nodal explants of grapevine

Combinations of BAP at 1.0 and 2.0 mg/l with IAA at 0.25, 0.5 and 1.0 mg/l, showed significant differences on the percentages of explants with shoots ($p \leq 0.05$), number of shoots per explants ($p \leq 0.05$) and number of nodes per shoot ($p \leq 0.01$). The best explants with shoots, number of shoots per explant and number of nodes per shoot were obtained on BAP at 2.0 mg/l with IAA at 0, 0 or 0.25 mg/l. However, the explants with shoots, number of shoots per explant and number of nodes per shoot

decreased with the increase in concentrations of IAA (Table 3) This finding is in contrast with that reported by Han *et al*. (2003) who found that a combination of 0.3 µM IAA and 4.4 µM BA gave the highest shoot growth and multiplication. In addition, Mederos (2005) reported that the best shoot multiplication was achieved with a woody plant (WP) medium with BAP and IAA or NAA. Earlier studies of Mhatre *et al*. (2000) reported that IAA not only induced roots, but also eliminated tufted shoots and calli in *Vitis vinifera*.

Table 3. Morphogenesis of nodal explants of grapevine on MS medium supplemented with combinations of BAP and IAA, after six weeks.

	IAA (mg/l)				
BAP	Explant with shoot (%)				
(mg/l)	0.0	0.25	0.5	1.0	Mean
1.0	80	70	70	80	75
2.0	100	100	80	50	82
Mean	90	85	75	65	
	No. of shoots per explant				
1.0	0.8	0.7	0.7	0.8	0.8
2.0	1.1	1.0	0.8	0.5	0.9
Mean	1.0	0.9	0.8	0.7	
	No. of nodes per main shoot				
1.0	1.4	1.2	1.3	0.8	1.1
2.0	3.1	3.3	1.5	0.9	2.1
Mean	2.3	2.3	1.4	0.9	

Callus induction was observed on all combinations of BAP and IAA. The highest callus formation was obtained on MS medium at 1.0 mg/l BAP combined with 1.0 mg/l IAA. Previous studies of Kim and Seon-Kyu (2002) reported that IAA at 2 mg/l induced callus from stem and petiole segments while NAA resulted in rooting of grapevine Sheridan cultivar.

Effect of kinetin on morphogenesis of nodal explants of the local grapevine cultivar

A single shoot was regenerated per explant on MS medium supplemented with different concentrations of kinetin (0.0, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 mg/l). The nodal explants showed a weak response to kinetin application. The percentage of explants with shoots, number of shoots per explant, number of nodes per shoot and height of main shoot were comparable at all concentrations of kinetin (Table 4). All concentrations of kinetin tested did not play a significant role in shoot induction or shoot multiplication from nodal explants of the local grapevine cultivar. Shoot regeneration was delayed in all concentrations of kinetin. Ibanez *et al*. (2003) reported that kinetin had a poor effect on sprouting or formation of multiple shoots from axillary buds.

Table 4. *In vitro* morphogenesis of nodal explants of grapevine on MS medium supplemented with different concentrations of kinetin, after six weeks of incubation.

Kinetin (mg/l)	Explants with shoots (%)	No. of shoots per explant	No. of nodes per main shoot	Height of shoot (cm)
0.0	50	0.5	1.1	1.0
0.25	60	0.6	1.1	1.2
0.5	42	0.42	1.1	1.3
1.0	42	0.42	1.3	1.0
2.0	50	0.5	0.7	1.4
4.0	58	0.58	1.3	1.5
6.0	50	0.5	1.1	1.2
8.0	42	0.42	1.3	1.0
SE(±)	7	0.1	0.3	0.2
Sign.	NS	NS	NS	NS

NS = not significant.

Effect of 2iP on morphogenesis of nodal explants of the local grapevine cultivar

Morphogenesis of nodal explants of the local grapevine cultivar showed poor responses to 2iP (Table 5). A single shoot per explant was developed using different concentrations of 2iP (0.0, 1.0. 1.5, and 2.0 mg/l). Percentage of explants with shoots, number of shoots produced per explant, number of nodes per shoot and length of main shoot were almost similar on all concentrations of 2iP after six weeks. Previous studies by Ibanez *et al*. (2003) showed that 2iP or kinetin only resulted in the production and growth of one shoot per sprouted bud.

Table 5. Morphogenesis of nodal explants of grapevine on MS medium supplemented with different concentrations of 2ip, after six weeks.

2ip (mg/l)	Explants with shoots (%)	No. of shoots per explant	No.of nodes per main shoot	Height of main shoots (cm)
0.0	90	0.9	1.8	2.0
1.0	100	1.0	1.6	1.7
1.5	80	0.8	1.7	1.8
2.0	100	1.0	2.5	2.3
SE	8	0.1	0.4	0.3
Sign.	NS	NS	NS	NS

NS = not significant.

Combinations of 2iP (1.0 and 2.0 mg/l) with IBA (0.0, 0.1, 0.3, and 0.5 mg/l) were comparable on percentage of explants with shoots and number of shoots per explant (Table 6). The explants with shoots and number of shoots per explant decreased significantly with the increase in concentrations of IBA from 0.0 to 0.5 mg/l (Tables 6). Some callus was induced at the base of explants on higher concentrations of IBA with 2iP.

Table 6. *In vitro* morphogenesis of nodal explants of grapevine on MS medium supplemented with different combinations of 2iP and IBA, after six weeks.

2iP(mg/l)	IBA (mg/l) Explants with shoot (%)				
	0.0	0.1	0.3	0.5	Mean
0.1	90	80	50	50	68
2.0	100	80	60	50	73
Mean	95	80	55	50	
	No. of shoots per explant				
0.1	1.4	0.8	0.5	0.3	0.8
2.0	1.8	0.9	0.5	0.4	0.9
Mean	1.6	0.9	0.5	0.4	

Effect of NAA on *in vitro* rooting of grapevine

Roots were formed on all shoots of grapevine on MS medium supplemented with NAA at 0.0, 0.25, 0.5 and 1.0 mg/l. Cultures in large vessels induced higher percentage of explants with roots compared with those grown in test tubes. Higher numbers of roots per explant were obtained on MS medium supplemented with NAA at 0.25 and 0.5 mg/l after six weeks (Table 7). Many authors reported that root formation occurred on MS medium with IBA (Heloir *et al*., 1997; Mhatre *et al*., 2000; Muhammad *et al*., 2008; Banilas and Korkas., 2007).

Table 7. Effect of different concentrations of NAA and type of containers on the rooting of *in vitro* shoots of grapevine, after six weeks.

Container type	NAA (mg/l) Explants with shoot (%)				
	0.0	0.25	0.5	1.0	Mean
Type tubes	50	80	70	80	70
Large vessels	90	90	90	80	88
Mean	70	85	80	80	
	No. of shoots per explant				
Type tubes	1.3	3.9	3.3	2.8	3.1
Large vessels	2.4	4.3	4.1	2.2	3.3
Mean	1.9	4.1	3.7	2.5	

In conclusion, rapid clonal micropropagation of the local grapevine cultivar by using nodal segments with axillary buds provides certain advantages. It is simple and requires low levels of growth regulators (cytokinins). BAP proved highly effective among the other tested cytokinins (kinetin and 2iP) for shoot induction. The best shoot production was obtained at 1.0 or 1.5 mg/l after six weeks. Combinations of BAP with IAA or 2iP with IBA did not improve shoot production. The best rooting was achieved on MS medium supplemented with NAA at 0.25 - 0.5mg/l.

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أثر السيتوكينينات و الأكسينات علي الإكثار النسيجي لسلالة من العنب البلدي باستخدام البراعم العقدية

[1] وعبدالمنعم محمد سلامة[2]ومحمد أحمد علي أحمد[1]عثمان عبد الله علي

كلية الزراعة والموارد الطبيعية، جامعة الجزيرة، وادمدني، السودان.[1]
هيئة البحوث الزراعية، وادمدني، السودان.[2]

الخلاصة

أجريت هذه الدراسة بوحدة زراعة الأنسجة النباتية بهيئة البحوث الزراعية بواد مدني، السودان، خلال الفترة 2006-2007 م. كان الهدف من هذه الدراسة إكثار السلالة البلدية لكرمه العنب عن طريق تقنية الإكثار النسيجي. أختبر أثر ثلاثة أنواع من السيتوكينينات في وسط موراشيجي واسكوق و هي بنزيل أمينوبيورين (BAP) و الكينيتين (kinetin) بالتركيزات 0.0 , 0.25 ،0.5 ،1.0 ،2.0، 4.0، 6.0 و8.0 ملجم/لتر وآيزوبنتينيل أدنين(2iP) و BAP بالتركيزات 0.0 , 1.0 , 1.5 و2.0 ملجم/لتر . كما تم اختبار تداخلها مع بعض الاوكسينات مثل اندول حامض الخليك (IAA) بالتركيزات 0.0 , 0.25 ، 0.5 و1.0 ملجم/لتر مع BAP بتركيز 1.0 أو2.0 ملجم/لتر و اندول حامض البيوترك (IBA) بالتركيزات 0.0 , 0.1 , 0.3 و0.5 ملجم/لتر مع 2iP بتركيز 1.0 أو2.0 ملجم/لتر . أختبر أثر منظم النمو نفثالين حامض الخليك (NAA) بالتركيزات 0.0 , 0.25، 0.5 و1.0 ملجم/لتر علي تحفيز التجذير لنبتات سلالة العنب البلدية المنتجة نسيجيا. اختلفت استجابة البراعم للإكثار النسيجي وذلك باختلاف منظم النمو ودرجة تركيزه. وكانت الاختلافات معنوية بين أنواع هذه المنظمات ودرجة تركيزها. أفضل استجابة لنمو البراعم كانت علي وسط موراشيجي واسكوق مضافا إليه BAP بتركيز 1.0 أو 1.5 ملجم/لتر مقارنة بمنظمات النمو الأخرى. كما إزدات أعداد النبتات النامية بزيادة فترة تحضين المنفصل النباتي المزروع حتى الأسبوع السادس. تسببت التركيزات العالية من BAP (2-8 ملجم/لتر) في إنتاج سيقان منتفخة و نمو غير طبيعي للأوراق. لم يؤدي تداخل IAA مع BAP إلي زيادة عدد النبتات النامية علي المنفصل النباتي المزروع . منظمات النمو kinetin و2iP لم تظهر أي فروق معنوية ما بين تركيزاتها المختلفة و كانت متساوية في عدد النبتات المنتجة وأعطت نبتة واحدة لكل منفصل نباتي. لم يحسن تداخل IBA مع 2iP في زيادة عدد النبتات النامية علي المنفصل النباتي المزروع. أفضل استجابة لنمو الجذور كانت علي وسط موراشيجي واسكوق مضافا إليه NAA بتركيز 0.25 أو0.5 ملجم/لتر بعد أربعه أسابيع من الزراعة.