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Propagation of Grape by Nodal Stem cuttings

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Abstract: Experiments were conducted to test the effect of MS salts concentration, potassium dihydrogen phosphate and growth regulators on shoot proliferation and growth in grapevine (*Vitis vinifera* L.), using explants derived from *in vitro* grown plantlets. Murashige and Skoog original medium was used as basal medium. Shoot proliferation occurred principally when using nodal stem segments as explants. Among MS-salt strengths tested, full MS- strength (1X) was optimum for shoot proliferation and elongation. Increasing the original phosphorus concentration in the medium by the addition of 170 mg/L KH_2PO_4 had a positive, marked effect on shoot number and shoot length. The nodal stem segment explants responded best to relatively low concentrations of NAA and kinetin, and a large number of shoots and good plantlets height were obtained on the medium containing 0.1mg/L NAA and 1.0 mg/L kinetin. Root formation occurred on the medium containing 0.3 mg/L IBA.

Key words: micropropagation; *Vitis* spp.; tissue culture

INTRODUCTION

Grape (*Vitis vinifera* L.) is a member of the family Vitaceae and is the most widely grown species of this family (Reisch and Pratt 1996). Grapes are native to Mediterranean climate but can be grown under irrigation in desert areas and tropical climate. It is one of the most important fruit crops grown in the world in terms of total acreage and production (FAO 2010). The cultivation of grapes in Sudan is limited. Yields are of low quality and quantity. The main single factor that limits the cultivation of grape in Sudan is the inaccessibility of stock mother plants of commercially known varieties. This has led to the scarcity of planting material. Importation of nursery transplants or even stem cuttings from abroad is difficult and expensive.

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Grapes are normally propagated for commercial cultivation by stem cuttings derived from dormant hardwood as a source. This method of propagation is rather troublesome, requiring special techniques and conditions (Hartmann *et al*. 1997). It is sometimes hampered by time consideration, cutting dormancy and space and is too slow to produce large number of plants in a short time, hindering the establishment of large-scale commercial vineyards. Additionally, the continuous use of stem cuttings for propagation has inadvertently led to detrimental spread of diseases within and between grape producing countries. Tissue culture techniques offer the advantages of rapid and large number of plants that are disease-free from a single stock plant on year-round basis (Blazina *et al.*1990; Mhatre *et al*. 2000).The potential of regeneration and plantlet production of grape has been realized (Mhatre *et al.* 2000; Salami *et al.* 2005). Propagation of some grape cultivars through regeneration from callus cultures (Bayir *et al.* 2007) and proliferation from shoot tips (Salami *et al*. 2005) is documented.

A variety of explant types have been used for culture initiation, including shoot tips (Ibanez *et al.* 2005; Salami *et al.* 2005), nodal segments (Jaskani *et al*. 2008), axillary buds (Shinde and Patel 2009) and leaf sections (Stamp *et al.* 1990). Not all grapevine species or cultivars respond in the same way to a generalized medium, and a strong genotype-dependent response to components of culture medium, with respect to growth and morphogenesis, has been reported (Gonzalez *et al*. 1996) and various grape cultivars respond differently to certain culture conditions (Qui *et al.* 2004). Tissue culture techniques result in rapid production of large number of planting material in a short time.

The objective of this research was to test the effects of concentrations of inorganic salts, potassium dihydrogen phosphate and hormonal addenda on *in vitro* propagation of grapevine with the aim of developing a rapid and reliable commercial technique for its mass clonal propagation.

MATERIALS AND METHODS

Actively growing shoots, approximately 15- cm long, were harvested from the vines of plants of "Biz-elanza" variety growing in the field of the Horticultural Department, Ministry of Agriculture and Forestry, Al-Mogran, Khartoum (Latitude. 35°15′N; Longitude. 33°32′E), Sudan. Leaves were removed and the stem segments were rinsed in running tap water for 30 min, disinfested by soaking in 2.63% Na OCl plus 1.0% Tween-20 for 15 min, and rinsed thrice in sterile distilled water for 1 min each. Primary explants consisting of single nodal stem segments and shoot tips, 1-2 cm long, were aseptically excised and inserted (one per tube, proximal portion down) into 25-x150-mm glass culture tubes containing a stock medium for initiation and establishment of cultures of herbaceous woody plant species. This medium consisted of Murashige and Skoog (1962) inorganic salts plus (per litre) 30 g sucrose, 40 mg myo-inositol, 40 mg adenine sulphate, 0.4 mg thiamine-HCl, 0.1 mg naphthalene acetic acid (NAA), 1.0 mg benzyl adenine (BA) and 7 g agar. The pH was adjusted to 5.7±0.1 by 1N NaOH or 1N HCl prior to the addition of agar. The medium was dispensed in 25 mL aliquots into 25-x150-mm glass culture tubes, capped with polypropylene caps, sterilized by autoclaving at 121°C and 1.01 kg/cm^2 present for 15 min and left to cool as slants in the culture room until use. All cultures were maintained in an incubation room at a constant temperature of 25±2°C and a 16-h light provided by Phillips cool-white (F4D) fluorescent tubes and an 8-h dark cycle.

Raised shoots *in vitro* were cut into nodal segments and repeatedly subcultured on a fresh batch of the stock plant medium, after each harvest of the newly formed shoots, for further multiplication and generation of additional stock plants for experimentation. Nodal stem segments and shoot tips were both used as explants in the first experiment where four levels of MS inorganic salt concentrations were tested at 0.25X, 0.5X, 1X or 2X, with 1X the normal concentration used in plant tissue culture, being the control. In the second experiment, the effects of increasing the concentration of phosphorus in the original MS medium, by the addition of 170 mg/L potassium dihydrogen phosphate (KH_2PO_4), on growth and

development of grapevine nodal stem segments was evaluated. In experiment 3, various combinations and concentrations of kinetin (0.0, 0.1, 0.3, 1.0 or 3.0 mg/L) and NAA (0.0, 0.01, 0.03, 0.1 or 0.3 mg/L) were added to the stock plant medium to test their effect on shoot proliferation and growth of cultured nodal cuttings. For rooting studies, *in vitro* produced shoots were placed on stock plant medium supplemented with 0.0, 0.01, 0.03, 0.1 or 0.3 mg/L indole-3-butyric acid (IBA), in experiment 4.

A completely randomized design was used with 10 single explants replicates for each treatment. Each tube was considered a replicate, and each experiment was repeated at least three times. Cultures were evaluated for number and length of shoots and roots and number of nodes after 6 weeks of incubation. Hand sections of new shoots were examined microscopically to evaluate axillary or adventitious origin. Data were subjected to the analysis of variance procedures using SAS (1990) statistical package, and Duncan Multiple Range Test was used to separate treatment means. The test was used where appropriate for mean comparisons.

RESULTS AND DISCUSSION

Explant type and MS-salt strength

In general, growth and development of micro-propagated plants depend on such factors as macro-elements composition, total salt strength of the medium and proper explants selection (Murashige 1974). There are many basic salt formulations available that were developed to satisfy the nutritional requirements of explants. The most common is the MS inorganic salt formulation of Murashige and Skoog (1962). Likewise, shoot apices and nodal stem segments have historically been recognized as the most widely used explants in all true-to-type vegetative propagations. The selection of a suitable explant for culture initiation is a crucial step for *in vitro* propagation of plants.

In this study, shoot tips and nodal segments containing axillary buds were cultured on media containing different MS-salt levels. Nodal segments

resulted in the highest values in all growth parameters measured displaying the greatest propensity for growth and development in culture compared with shoot tip explants (Table 1 and Fig.1). Among all MS-salt strengths tested, 1X significantly supported the largest number of shoots, the longest shoots, and the greatest number of nodes. The values of all the variables measured increased to a maximum with increasing MS-salt strength to 1X and then decreased substantially at 2X. The few shoots that were formed on the medium containing 2X MS-salt strength showed acropetal development of leaf necrosis. There was no significant difference in the number of nodes between nodal stem segments cultured on media containing 0.25X, 0.5X or 2X MS-salt strengths, whereas the lowest number of nodes was obtained with shoot tips cultured on 0.25X, 0.5X and 2X MS-salt strengths. Shoot tip explants gave the shortest shoots on all MS-salt strengths tested, while nodal cuttings recorded the shortest shoots on the medium containing 0.25X MS-salt strength.

These findings are in accord with those of Yu and Meredith (1986), Mahtre *et al*. (2000) and Jaskani *et al.* (2008), who found nodal stem segments are best for multiple shoot regeneration of a number of grapevine cultivars. More or less similar findings were reported by Yu and Reed (1995) for hazelnuts and Ara *et al*. (1997) for roses that nodal segments responded better to *in vitro* culture than did shoot tips. In contrast to these results were those reported by other investigators (Blazina *et al.* 1990; Ibanez *et al.* 2005 Salami *et al.* 2005) who obtained best proliferation rates using grapevine shoot tips as explants. Factors that may be responsible to these contradicting results include, but not limited to, differences in genotype, composition of nutrient media and incubation conditions.

Nodal stem segments were physiologically and ontologically older than shoot tips. They were more differentiated, with relatively higher number of well-developed axillary buds and more nutritive food reserve and other growth promoting substances, than the less differentiated and less developed shoot tips.

Table 1. Effect of MS salt concentrations on growth and development of grapevine nodal stem segments and shoot tips cultured *in vitro*, after 6 weeks of incubation period

MS-salt strength(X)	Nodal cuttings			Shoot tips		
	No. of shoots	No. of nodes	Shoot length(cm)	No. of shoots	No. of nodes	Shoot length(cm)
0.25	1.70cd	2.60bc	3.30bc	1.50cd	1.06d	1.80e
0.5	2.30bc	3.00b	4.00b	1.80cd	1.30d	2.80cd
1.0	4.20a	4.70a	5.70a	2.70b	2.20c	3.50bc
2.0	1.20d	2.30bc	2.20de	1.30d	1,03d	1.50e

Means in a column followed by the same letter(s) are not significantly different at P=0.05, according to Duncan Multiple Range Test.

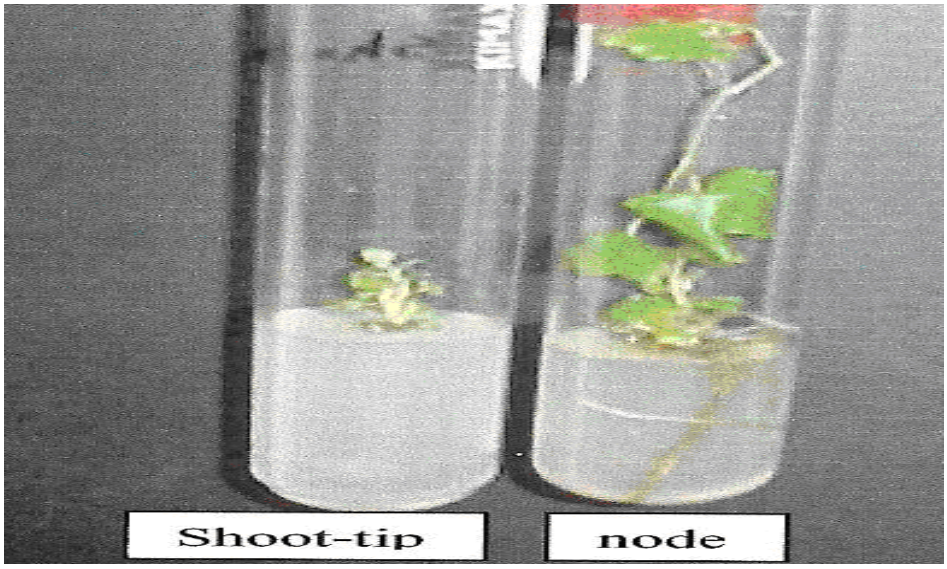

Fig.1. Growth and development of shoot tip explant (left) and nodal stem segment explant (right) of grapevine on stock plant medium, after 6 weeks from culture initiation

MS medium proved to be effective for tissue culture propagation of many plant species (Murashige 1974), and grapevine is not an exception (Ibanez *et al.* 2005; Jaskani *et al.* 2008). The suitability of the normal MS concentration used in this study for propagation of grapevine using nodal stem segments could be attributed to its high content of nitrates, potassium and ammonium. Some investigators (Zhang *et al.* 2006; Sajid and Zahoor 2008) advocated using dilute MS salt-strengths for successful culture initiation and shoot proliferation of grapevine, whereas Mukherjee *et al.* (2010) acknowledged the reduction of MS-salt nitrates to ½-strength to obtain high rates of shoot proliferation.

Potassium dihydrogen phosphate

The level of K and P ions in the original MS medium is low (Troncoso *et al.* 1999). Increasing the concentration of phosphorus of the original MS medium, by the addition of 170 mg/L potassium dihydrogen phosphate, markedly increased the number and length of shoots as well as the number of nodes of *in vitro* cultured grapevine nodal stem segments (Table 2). These results support the findings of Chin *et al*. (1988) who incorporated 170 mg sodium dihydrogen phosphate into an MS- salt based medium to culture isolated embryos of a variety of difficult-to-germinate seeds and those of Smith and Murashige (1970) who increased the concentration of phosphorus in MS medium for successful culture initiation and growth of apical meristems of a variety of plant species. Addition of phosphate higher than 170 mg/l for other plant species has been reported (Idris *et al.* 2006). The positive responses of *in vitro* cultured nodal segments of grapevine to the increase in the phosphate concentration of the original MS medium merits further studies.

Table 2. Effect of potassium dihydrogen phosphate (KH_2PO_4) on growth and development of grapevine nodal stem segments cultured *in vitro*, after 6 weeks of incubation period

Supplement	No. of shoots	No. of nodes	Shoot length (cm)
KH_2PO_4	5.20a	5.30a	7.50a
None	3.50b	4.20b	5.20b

Means in a column followed by the same letter are not significantly different at P=0.05, according to *t* test

Rapid phosphate uptake occurs at medium pH value of 4.0, declining progressively with increase in pH values of more than 4.0 (George and Sherrington 1984). The pH of the medium used in this study was adjusted to 5.7±0.1 which may perhaps be responsible for the lower concentration of available phosphate than optimal for growth and morphogenesis of cultured grapevine nodal stem segments. The supplementation of the original MS medium with an additional phosphate might have had a compensative effect. Further research is needed to determine optimal rates for phosphorus concentration as an effective medium additive for shoot proliferation and elongation in grapevine.

Growth regulators

The *in vitro* induction of morphogenesis is often controlled by the incorporation of an auxin and a cytokinin combination into the basal medium (Murashige 1974). Shoot proliferation, number of nodes and length of shoots were highest on the medium containing 0.1 mg/L NAA and 1.0 mg/L kinetin with no significant difference from those obtained on the medium containing 0.3 mg/L NAA and 3.0 mg/L kinetin (Table 3). However, there was a pronounced callusing and vitreous shoot formation tendency in this combination treatment.

These results are in line with those reported by others (Mahtre *et al*. 2000; Zhang *et al*. 2006; Jaskani *et al.* 2008) that better responses of grapevine are obtained by use of relatively low levels of growth regulators in clonal propagation media of grapevine. The present results also confirmed the findings of Jaskani *et al.* (2008) who demonstrated that relatively high levels of growth regulators may enhance callus formation and promote hypersensitivity.

Neither NAA nor kinetin alone was able to promote multiple shoot formation and elongation. There appears to be a synergistic effect between NAA and kinetin, and both were needed to maximize shoot proliferation from grapevine nodal stem segments. The data revealed that a shoot multiplication medium containing 0.1 mg/L NAA and 0.1 mg/L kinetin can be used to establish proliferating shoot culture by repeatedly subculturing original nodal stem segments on the same medium after each

harvest of new shoots in a similar manner to the findings of Zhang *et al.* (2006). Histological observations showed that shoot proliferation resulted from lateral shoot development and not from basal callus tissue. The chance for somaclonal variation here would likely be low, because multiplication was obtained on medium containing low concentrations of growth regulators.

Indole-3-butyric acid

Induction of roots was the principal morphogenic pattern in this experiment. Shoots proliferated on kinetin-containing medium need to be subcultured on IBA-supplemented medium for rooting. Root formation was frequent in most of the tested concentrations of growth regulator combinations, but these roots were few in number and fragile (Data not presented).

The effect of different concentrations of IBA on root induction on *in vitro* produced grapevine shoots is portrayed in Table 4. With a constant level of kinetin (1.0 mg/L) in the stock plant medium, the values of all growth variables measured, with the exception of number of shoots, increased significantly with increasing IBA concentration from 0.01 to 0.1 mg/L. Concentrations of IBA greater than or equal to 0.1 mg/L significantly increased root formation and elongation. The highest number of roots and longest roots per explant were obtained on the medium containing 0.3 mg IBA/L. All IBA concentrations tested had no effect on shoot number but stimulated shoot elongation. The longest shoots per explant were obtained at concentrations of IBA equal to or greater than 0.1 mg/L. IBA may have augmented natural apical dominance, hence suppressing shoot formation and accounted for the increased length of the formed few shoots. Concentrations of IBA greater than or equal to 0.1 mg/L caused the formation of thicker roots and few secondary roots, whereas concentrations less than 0.1 mg/L promoted the development of roots with normal appearance and numerous secondary roots and root hairs. The results confirmed earlier reports (Lee and Wetzstein 1990; Jaskani et al. 2008) that IBA is necessary for *in vitro* rooting of grapevine shoots. A strong genotype-dependent response to rooting media and to auxin type and concentration among grapevine cultivars exist (Gonzalez *et al*. 1996).

A combination of auxins (Lewandowski 1991), NAA (Gray and Benton 1991), IBA, NAA or IAA (Zhang *et al.* 2006), IAA (Mukherjee *et al*. 2010) and even an auxin-free medium (Salami *et al.* 2005) have been used for rooting of *in vitro* produced grapevine shoots.

In conclusion, the importance of the clonal propagation protocol of grapevine herein reported may be related to the lack of a callus intermediate step. Nodal segments appear to be suitable explants for rapid clonal propagation of grapevine. The multiplication rate obtained is of practical interest, since it was achieved from freshly cultured explants after 6 weeks of culture and could be enhanced further with repeated subculture under a mass production strategy. It may be possible to maintain stock *in vitro* for extended periods, and to propagate these plants on a year-round basis. Additional research is warranted to improve *in vitro* rooting and to analyze acclimatization and establishment under greenhouse or shaded conditions before this protocol can be considered an economic alternative to the conventional methods of vegetative propagation of grapevine.

Table 3. Effect of NAA and kinetin on growth and development of grapevine nodal stem segments cultured *in vitro*, after 6 weeks of incubation period

NAA (mg/L	Kinetin (mg/L	No. of shoots	No. of nodes	Shoot length (cm)
0.0	0.0	1.20cde	2.20ghi	1.80ghi
	0.1	1.50bcd	2.30ghi	1.70ghi
	0.3	1.30cde	2.50ghi	2.00fghi
	1.0	1.20cde	2.50ghi	2.10fgh
	3.0	1.20cde	2.70fghi	2.30ef
0.01	0.0	1.00de	2.20ghi	1.30j
	0.1	1.50bcd	3.70ghi	2.30ef
	0.3	1.20cde	3.00fghi	2.20ghi
	1.0	1.00de	3.00fghi	2.00fghi
	3.0	1.30cde	3.10fghi	2.10fgh

Table 3.(contin)

NAA (mg/L	Kinetin (mg/L	No. of shoots	No. of nodes	Shoot length (cm)
0.03	0.0	1.00de	1.70i	1.30j
	0.1	1.20cde	1.80i	2.00fghi
	0.3	1.50bcd	1.90i	2.20ghi
	1.0	1.20cde	2.00hi	2.30ef
	3.0	1.20cde	3.50bcd	2.60de
0.1	0.0	1.30cde	1.70i	1.70hi
	0.1	1.70bcd	3.70defg	3.00d
	0.3	4.00ab	5.00bcd	3.10cd
	1.0	4.50a	6.80a	4.30b
	3.0	1.00de	6.20ab	4.00b
0.3	0.0	0.83e	3.00fghi	1.80ghi
	0.1	0.86e	4.20cdef	3.00d
	0.3	1.20cde	4.80bcde	3.00d
	1.0	1.30cde	5.00bcd	3.00d
	3.0	2.50a	5.80abc	6.00a

Means in a column followed by the same letter(s) are not significantly different at P=0.05, according to Duncan Multiple Range Test

Table 4. Effect of IBA on growth and development of grapevine nodal stem segments cultured *in vitro*, after 6 weeks of incubation period

IBA (mg/L)	No. of shoots	No. of nodes	No. of roots	Root length (cm)	Shoot length (cm)
0.0	1.00a	3.00b	2.00c	1.20c	1.70c
0.01	1.00a	3.20b	2.20c	1.50c	3.00b
0.03	1.00a	3.30b	2.50b	1.90b	3.00b
0.1	1.00a	4.80a	5.20a	2.30ab	5.20a
0.3	1.00a	5.20a	6.80a	2.80a	5.50a

Means in a column followed by the same letter(s) are not significantly different at P=0.05, according to Duncan Multiple Range Test.

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إكثار العنب بالعقل الساقيه العقديه

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قسم زراعة الانسجه، هيئة التقانه الحيويه والهندسه الوراثيه، المركز القومى للبحوث، ص. ب. 2404 الخرطوم، السودان.

المستخلص: أُجريت تجارب لإختبار تأثير تركيز املاح وسط "موراشيقي" و"اسكوج"، وإضافة فوسفات البوتاسيوم ثنائي الهيدروجين، منظمات لنمو لى تكوين ونمو السيقان في العنب (*Vitis vinifera* L.) بإستخدام قمم سيقان وعقل سيقان عقديه، كاجزاء إستزراع، مأخوذه من نبيتات مزروعه في الانابيب. أُستخدم وسط "موراشيقي واسكوج" الاصلي كوسط اساسي. تم تكوين السيقان بصوره رئيسه عند إستخدام عقل الساق العقديه كاجزاء إستزراع، وثبت ان تركيز الاملاح الكامل (1X) هو الأمثل لتكوين السيقان وإستطالتها. وكان لزيادة تركيز الفسفور الاصلي في وسط MS الغذائي بإضافة 170 ملجم KH_2PO_4/لتر تأثيراً إيجابياً واضحاً على عدد السيقان وعلى طولها. إستجابت عقل الساق العقديه بصورةٍ أفضل للتراكيز المنخفضه نسبياً من نافثالين حمض الخليك (NAA) والكينتين، حيث تم الحصول على أكبر عدد من السيقان وأفضل طول للنبيتات على وسط غذائي يحتوى 0.1 ملجم NAA /لتر و 1.0 ملجم كينتين/لتر. تكونت الجذور على وسط غذائي يحتوي 0.3 ملجم اندول حمض البيوتيريك (IBA) /لتر.

[1] قسم البساتين، كلية الدراسات الزراعيه،جامعة السودان للعلوم والتكنولوجيا، شمبات ، السودان