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Clonal Propagation of *Ziziphus spina-christi* by Shoot Tip Culture: I. Improved Inorganic and Organic Media Constituents for *in vitro* Shoot Multiplication

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Abstract. Cider is an important cultivated tree and one of the few truly native tree species of Saudi Arabia that is still growing along with many newly introduced exotic plants. The present study describes the influence of inorganic and organic media constituents on micropropagation of the Noaf variety, *Ziziphus spina-christi.* Shoot apices of 0.5 - 1.0 cm were cut and transferred to a basal tested nutrient medium. The full concentration of M & S in this study yielded the best results in shoot length (3.02 cm) and number of leaves (1.80). No bud growth was observed in media devoid of sucrose. Shoot length, number of nodes and number of leaves were improved steadily with the addition of sucrose up to the concentration of 3.0% which showed a significant increase in all studied growth parameters. The full concentration of White's organics (1X), gave a significant increase in the shoot length (1.6cm) and number of nodes (2.4) over all other treatments. The bud growth of Cidir showed little response to the inositol treatment, since no significant difference in shoot length was observed between the control treatment (no inositol) and 1000 mg/L. The best adenine sulphate concentration that gave the best enhancment in most growth parameters was 80 mg/L. Shoot length (5.56 cm), number of nodes (6.7) and number of leaves (3.2) were enhanced by 1 mg/L charcoal concentration. This treatment was not significantly different from 2 mg/L treatment in shoot length and number of nodes although the 1 mg/L treatment showed a noticeable increase in these two parameters. Tissue culture application to the clonal propagation of "Cidir" will allow for the elimination of diseases and the rapid clonal production in large numbers of genetically identical plant material. The technique also greatly enhances the introduction of this variety (Noaf) and stimulates interest among nurserymen in Cidir cultivation as a fruit tree. Establishment of an *in*

vitro propagation scheme would enhance cloning of "Cidir", since propagules for future propagation can be derived from plantlets growing *in vitro*, and circumventing the requirements for explants from this sole stock plant available.

Introduction

Ziziphus spina-christi (L.) Desf., locally known as Cidr, is a multipurpose tree species belonging to the botanical family Rhamnaceae. It is an important cultivated tree and one of the few truly native tree species of Saudi Arabia that is still growing along with many newly introduced exotic plants (Mandavillae, 1990). It is considered one of the most drought-resistant fruit crops adapted to the environmental conditions of Saudia Arabia. The tree is growing wild on a wide range of soil types in the southern and south-western region and has been abundantly cultivated as an irrigated ornament and shade tree in the streets and backyards of many private homes, schools, hospitals, and government premises throughout the Kingdom. The tree is basically evergreen but it looses some of its leaves during the winter and sometimes summer months. It is hard, and can adapt and grow in a wide range of temperature from below 0^{o}C to 52^{o}C. The trees are inexpensive to grow for no irrigation is needed once the tree is established. Two growth cycles have been determined (Abo-Hassan and El-Osta, 1983).

Ziziphus is commonly propagated by seeds and therefore exhibits a wide genetic heterogeneity. This genetic variability may benefit Ziziphus in harsh environments by allowing rapid adaptation to changing climatic conditions. The tree is cross-pollinated and highly outbred (Sudhersan and Hussain, 2003). Germination is epigeal and seedling growth rate is medium, reaching a suitable size (40–50 cm in length) for planting after 4–5 months in the nursery.

Despite the fact that *Ziziphus spina-christi* is propagated from seed, there is a need for vegetative propagation when superior genotypes are found. Plant tissue culture offers the possibility of rapid clonal propagation which provides potential for large scale production of genetically identical superior strains for commercial use (Sharp, *et al*., 1980). Propagation of plants through shoot tip culture allows recovery of genetically stable and true-totype progeney (Barakat and El-Lakany, 1992; and Barakat, 2008).

The proliferation of axillary shoot from cultured shoots apices and nodal segments is greatly influenced by the nature of the culture medium used. Most media formulations for the tissue culture of many woody species have generally involved the use of Murashige and Skoog (M & S) (Murashige and Skoog, 1962) basal medium or at least, the use of its salt mixture as a starting point for medium development. More diluted concentrations of M & S macronutrients may prove to be better for woody plant tissues. Reduction may involve one ion (Durzan, *et al.*, 1973), more ions (Washer, *et al*., 1977) or simply employment of a reduced concentration of M & S salt mixtures (Werner and Boe, 1980; and Pliego-Alfaro, *et al.*, 1987). Hence, it becomes essential to optimize culture conditions for a particular clone/ cultivar/ rootstock or newly bred line that needs large scale planting, but availability of sufficient planting stock is a limitation. The present manuscript describes influence of inorganic and organic media constituents on micropropagation of the Noaf variety, *Ziziphus spina-christi.*

Materials and Methods

Actively growing shoot apices 4 to 5 cm long were obtained from a selected Cidir (*Zizyphus spina-christi* Wild) 5 years old tree growing in the vicinity of King Saud University at the Staff and Employees Housing Quarter. This single tree was selected on the basis of its heavy yield and fruit characteristics. It served as the only source of all experimental explants throughout this study. The leaves were removed except the 6 or 8 youngest ones enclosing the shoot apex. Explants were soaked in distilled deionized water prior to surface sterilization treatments. Explants of 3 cm long were then surface sterilized for 15 minutes with 2.63% w/v sodium hypochlorite (NaOCl), containing two drops of Tween-20 emulsifier per 100 ml solution. After rinsing three times with autoclaved distilled deionized water in a laminar-air-flow hood, 1- 1.5 cm terminal growth were excised with a surgeon's scalpel equipped with a No.11 blade. Careful attention was made to ensure a smooth cut at the base of the isolated shoot apices. Following excision, 0.5- 1.0 cm shoot apices were cut and transferred to a basal nutrient medium, containing Murashige and Skoog (1962) inorganic salt mixture, and the following components in mg/L: NaH_2PO_4. $2H_2O$, 170; sucrose, 30,000; i-inositol, 100; thiamine-HCl, 1.0; pyridoxine-HCl, 0.5; nicotinic acid, 0.5; glycine,

2 and Bacto agar, 7000. The pH was adjusted to 5.7 prior to addition of agar. The medium was then dispensed in 25-ml aliquots into 25- x 150-mm culture tubes and each tube was capped with a Bellco kaput and autoclaved for 15 minutes at 1.01 kg and 121^{o}C. Tubes were cooled slanted at 45^{o}C.

Shoot apices were initially cultured on the basal medium for two weeks before being recultured onto the requisite test medium. All experimental media consisted of the constituents of the basal medium except for the component under test. The following media components have been tested with the objective of tailoring the nutrient medium to support a high proliferative rate of maxillary shoot formation on the cultured explants.

A comparison between Murashige and Skoog (MS 1962) inorganic salt formulation and Whites (1943) salt formulation was done, except for the iron which was added according to Said and Murashige (1979). Sucrose is the most used source of energy in plant tissue culture. Different concentrations of sucrose were used (0.0, 0.75, 1.5, 3.0 and 6.0%). Modified Whites organics (Mahdi, 1985) were tested with different concentration (0X, 0.5 X, 1X, 5X and 10X). Adenine sulphate (0, 40, 80, 160, 320 mg/L) was also tested in medium. Different concentration of inositol(0.0, 40, 100, 400, 1000 mg/L) were tested. Activated charcoal (0, 0.5, 1, 2, 4%) was also tested in standard rooting medium.

All cultures were incubated in a temperature and light controlled room at 27^{o}C ± 2^{o}C under 16 hours daily exposure to 1000 lux, Grow-Lux light. Ten to twenty excised shoot apices were planted per treatment in each experiment. Shoot apices were recultured every four weeks at which time data were taken.

In vitro traits such as number of axillary shoots, number of roots, number of leaves, number of nodes and length of axillary shoots were recorded. A completely randomized design (CRD) was used in this study with 10 replications according to El-Nakhlawy (2008). Data were analyzed by the General Linear Models procedure of the Statistical Analysis System (SAS Institute, 2000). LSD test at $p \leq 0.05$ was used to test the significance between the means.

Results and Discussion

Effect of Murashige and Skoog's Salt Medium on in Vitro Traits

Enhancement of shoot length, number of nodes and number of leaves was achieved by full concentration of Murashige and Skoog's salt medium (10 mg/L). The superority of this treatment over all other concentrations was clear and statistically significant (Table 1). However, no significant difference was found between 10 mg/L and 5 mg/L in shoot length and similarly the number of nodes. The 2.5 mg/L treatment was found effective in increasing the number of nodes and was not statistically different from 10 and 5 mg/L treatments. Very limited growth was achieved by double M & S salt (Table 1). The Murashige and Skoog medium and its modifications classified as high salt media are most used in tissue culture of woody plants, chestnut (Vieitez and Vietez, 1980), Dallbergia and Eucalyptus, (Mascarenhas, *et al*., 1982b), Euacalyptus, (McComb and Bennette, 1982) and some tropical and subtropical fruit trees (Litz and Jaiswal, 1991).

The full concentration of M & S in this study yielded the best results in shoot length (3.02 cm) and number of leaves (1.80). This result is supported by many previous findings which are well documented in the literature (Said, 1978; Douglus, 1984; Welander, 1985; and Idris, 1994). The full concentration of M & S salt was thus choosen for Cidir culture throughout this study.

Table 1. Effect of different concentrations of M & S on the growth of excised Cidir shoot tips.

Character Treatments	Shoot length (cm)	Number of nodes	Number of leaves
0.00 X	0.54 c	1.00 c	0.01 c
0.25 X	1.82 b	2.60 ab	0.60 bc
0.50 X	2.70 a	3.20 a	1.40 ab
1.00 X	3.02 a	3.40 a	1.80 a
2.00 X	1.52 b	2.00 b	1.00 ab
LSD (0.05)	0.61	0.98	0.80

Means with the same letter(s) within each column are not significantly different at $p \leq 0.05$

Many investigators working on the propagation of woody plants found that reduced concentrations of M & S salts gave better results (Lee and Rao, 1986; Werner and Boe, 1980; and Pliego-Alfaro, *et al*., 1987).

The results of this study also revealed that there was no significant difference between one half and full concentration of M & S in all studied parameters, although half the concentration gave lower values than full M & S concentration. As for the number of nodes, one fourth, one half and full M & S concentrations showed no significant differences among them, indicating the different response of the *in vitro* traits.

Effect of Sucrose on in Vitro Traits

No bud growth was observed in media devoid of sucrose. Shoot length, number of nodes and number of leaves were improved steadily with the addition of sucrose up to the concentration of 3.0% which showed a significant increase in all *in vitro* traits. Sucrose concentration of 4.5% and more caused a significant decline in the above mentioned parameters, this decline was clearer in the 6.0% concentration. The data indicated that 3.0% treatment was the best (2.75cm, 3.00 and 3.00 for shoot length, number of nodes and number of leaves, respectively), since it encouraged more lateral branches with vigorous growth (Table 2). It is essential to supplement the tissue culture media with a source of energy, since it supports an excised plant tissue. The sucrose is the main mostly used source of energy in tissue culture media (Murashige and Skoog, 1962). The 3.0% sucrose concentration of the basic M & S medium which is universally applied, was found to be the best concentration in this study. The optimal concentration of sucrose varies according to the plant type. Low concentrations, 20 g/L was used for the proliferation of peach (Miller, *et al*.,1982); *Pinus taeda* (Mehra-palta, *et al*., 1977), and Eucalyptus (Gupta, *et al.,* 1983). Higher concentration was employed for banana and plantain multiplication medium (Cronauer and Krikorian, 1984).

The developmental stages of tissue may require different concentration of sucrose (Neiderweiser, *et al*., 1990), as they rescued Ornithogalum embryos on a medium supplemented with 7% sucrose and devoid of hormones. They also obtained better growth of these embryos in a medium with as low sucrose concentration as 1%. The results of this study showed that a decline in all the studied growth parameters was obtained in a sucrose concentration of 6.0% and above. This decline could be explained by the effect of osmotic pressure exerted by higher concentration on shoot tip growth and development (Gautheret, 1955).

Effect of White's Organics Medium on in Vitro Traits

The full concentration of White's organics (1X=10.0 mg/L), gave a significant increase in the shoot length and number of nodes (2.75 cm and3.00, respectively) over all other treatments. The concentration 1.0 X or more resulted in a fewer leaves than the control (Table 3). Media lacking White's organics or having the lowest W_0 concentration (0.5 X) were found to favour the increase in length and number of lateral branches (Table 3). Results of Table 3 also revealed that the growth vigor of Cidir shoot tips was similar through all studied White's organics concentrations.

Table 2. Effect of different concentrations of sucrose on the growth of excised Cidir shoot tips.

Character Treatments	Shoot length (cm)	Number of nodes	Number of leaves	Number of branches	Vigor
0.0 %	0.00 e	0.00 e	0.00 e	0.0	0.0
0.5 %	2.35 b	2.30 b	2.70 b	0.57 ±0.2*	4.1 ±0.55
3.0 %	2.75 a	3.00 a	3.00 a	1.1 ±0.1	4 ±0.38
4.5 %	1.95 c	2.00 c	2.40 c	0.11 ±0.11	2.25 ±0.25
6.0 %	1.15 d	1.62d	1.55 d	0.1 ±0.1	2.12 ±0.26
LSD(0.05)	0.15	0.27	0.23		

Means with the same letter(s) within each column are not significantly different at p ≤0.05.
*± Standard error.

Table 3. Effect of different concentrations of White's organics on the growth of excised Cidir shoot tips.

Character Treatments	Shoot length (cm)	Number of nodes	Number of leaves	Number of branches	Vigor
0 X#	1.17 c	2.70 b	0.2 ±0.2*	0.5 ±0.0	3.5 ±0.5
0.50 X	1.33 b	2.80 b	0.2 ±0.2	0.3 ±0.1	3.6 ±0.37
1.00 X	1.60 a	3.40 a	0.0	0.0	3.8 ±0.25
5.00 X	1.31 b	2.70 b	0.0	0.0	3.8 ±0.25
10.0 X	1.18 b	2.60 b	0.0	0.0	2.9 ±0.26
LSD(0.05)	0.15	0.42			

Means with the same letter(s) within each column are not signifcantly different at p ≤0.05.
* ± Standard error.
# X = 10 mg/L.

Effect of Inositol Treatment on in Vitro Traits

The bud growth of Cidir showed little response to the inositol treatment since no significant difference in shoot length was observed between the control treatment (no inositol) and 1000 mg/L. A noticeable reduction in shoot length, number of nodes and number of leaves was reported in 100 mg/L inositol concetration (Table 4). However, higher number of leaves was obtained in media containing 400 mg/L inositol and 1000 mg/L concentrations while the increased number of nodes (5.20) was in media containing the highest inositol concentration (1000 mg/L). Number of lateral branches and their length showed no consistence and no statistically significant response to the increasing concentrations of inositol (Table 4). The effect of i-inositol vitamin in the culture media was inconsistent in the literature. Murashige (1974) showed that i-inositol was not necessary in the culture media, whereas Curir, *et al.*, (1986) and Zhang and Stoltz (1989) recommended the use and stressed on the importance of i-inositol in the culture media. The results of this study on Cidir shoot tips support Murashige observation, although the higher concentrations, 400 and 1000 mg/L, resulted in higher number of leaves and nodes respectively. The recommendations of this study are in line with that of Murashige (1974).

Table 4. Effect of different concentrations of Inositol on the growth of excised Cidir shoot tips.

Character Treatments	Shoot length (cm)	Number of nodes	Number of leaves	Number of branches	Vigor
0 Mg/L	4.30 a	4.60 b	3.20 b	0.7 ±0.3*	0.81 ±0.2
40 Mg/L	4.40 a	4.70 b	3.00 b	0.1 ±0.1	0.1 ±0.1
100 Mg/L	4.24 a	4.30 b	2.60 c	0.6 ±0.3	0.9 ±0.35
400 Mg/L	4.50 a	4.70 b	3.60 a	0.4 ±0.2	0.68 ±0.22
1000 Mg/L	4.12 a	5.20 a	3.60 a	0.3 ±0.2	1.1 ±0.6
LSD(0.05)	0.46	0.45	0.39		

Means with the same letter(s) within each column are not significantly different at $p \leq 0.05$.
* ± Standard error.

The 1X vitamins in this study resulted in an increase in shoot length and number of nodes which indicates the necessity of vitamin supplement in the Cidir culture media. This recommendation supports many previous investigations like Murashige (1974); Soozek and Hempel (1988); and Idris (1994). It is worthy to note that lack of modified White's organics or lower concentrations (0.5X) in the media significantly increased both the number and length of the lateral

branches. A similar observation was reported by Zhang and Stolts (1989) working on the propagation of *Euphorbia fulgares*, but due to the effect of another vitamin (inositol). The same effect of inositol was reported on tissue culture of Gerbera plant (Soozek and Hempel, 1988).

Effect of Adenine Sulphate on in Vitro Traits

The best adenine sulphate concentration that gave the best enhancement in most *in vitro* traits was 80 mg/L. The exception was the shoot length which was higher at zero mg/L treatment (3.15 cm). The 40 and 80 mg/L treatments resulted in higher number of nodes (4.30) and number of lateral branches (0.4). Concentrations above 80 mg/L resulted in a reduced number of nodes, number of leaves and number of lateral branches, and this reduction continued over 160 and 320 mg/L treatments (Table 5). Another material that has an increasing effect on lateral shoot number is the nitrogenous base adenine sulphate and some other phenolic compounds such as phloroglucinol which activate growth and apical bud proliferation (Jones and Hatfield, 1976; and Hunter, 1979).

Table 5. Effect of different concentrations of adenine sulphate on the growth of excised Cidir shoot tips.

Character Treatments	Shoot length (cm)	Number of nodes	Number of leaves	Number of branches
0 mg/L	3.15 a	3.50 b	2.60 c	0.2 ±0.13*
40 mg/L	2.68 b	4.30 a	3.00 b	0.4 ±0.16
80 mg/L	2.97 a	3.80 b	3.30 a	0.4 ±0.22
160 mg/L	2.29 c	3.30 c	3.00 b	0.2 ±0.13
320 mg/L	2.30 c	3.20 c	3.00 b	0.0
LSD (0.05)	0.20	0.47	0.28	

Means with the same letter(s) within each column are not significantly different at $p \leq 0.05$
* ± Standard error.

The results of this study showed an increasing effect in number of lateral branches at concentrations of 40 and 80 mg/L adenine sulphate. Paek and Han (1988) reported more rapid growth, multiplication and production of gloxinia lateral branches in media supplemented with 40 mg/L adenine sulphate. Adenine sulphate at concentrations more than 80 mg/L in this study resulted in a deterious effect on all studied growth parameters. Similar supporting results were also reported by many investigators: Davies, *et al*. (1972); George and Sherrington (1984) and Samartin, *et al*. (1989).

Effect of Activated Charcoal on in Vitro Traits

Shoot length, number of nodes and number of leaves were enhanced by 1 mg/L charcoal concentration. This treatment was not significantly different from 2 mg/L treatment in shoot length and number of nodes, although the 1 mg/L treatment showed a noticeable increase in these two parameters (Table 6). The data in Table 6 showed clearly that charcoal was ineffective on inducing lateral branching of Cidir shoot tip. Another research showed that activated charcoal had an effective role in absorption of unknown components, which were produced through chemical processes within the media. Sometimes these unknown components played the role of growth inhibitor at morphogenesis stages. In addition, the activated charcoal, which eliminated light and provided a reasonable physical environment for the rhizosphere, prevented undesirable callusing and helps rooting (Nissen and Sutter, 1990).

Table 6. Effect of different concentrations of Charcoal on the growth of excised Cidir shoot tips.

Character Treatments	Shoot length (cm)	Number of nodes	Number of leaves	Number of branches
0.0 mg/L	3.48 d	3.6 d	3.4 a	1.20 ±0.13*
0.5 mg/L	3.54 c	4.8 c	2.1 c	1.22 ±0.22
1.0 mg/L	5.56 a	6.7 a	3.2 a	1.12 ±0.12
2.0 mg/L	5.03 a	6.3 a	2.5 b	1.02 ±0.24
4.0 mg/L	3.61 b	5.4 b	2.4 b	0.80 ±0.13
LSD(0.05)	0.41	0.52	0.39	

Means with the same letter(s) within each column are not significantly different at $p \leq 0.05$.
* ± Standard error.

Tissue culture application to the clonal propagation of "Cidir" will allow for the elimination of diseases and the rapid clonal production in large numbers of genetically identical plant material. The technique also greatly enhances the introduction of this variety (Noaf) and stimulates interest among nurserymen in Cidir cultivation as a fruit tree. Establishment of an *in vitro* propagation scheme would enhance cloning of "Cidir", since propagules for future propagation can be derived from plantlets growing *in vitro*, and circumventing the requirements for explants from this sole stock plant available. Hence, stock-independent *in vitro* procedure based on re-cycling of contaminant-free propagules could be developed on one hand. On the other hand, "Cidir" propagation *in vitro* could be carried out all-year-round avoiding the seasonal restrictions of stock plant growth. "Cidir" trees are either flowering or

fruiting, and this affects greatly the availability of explants for *in vitro* culturing. Stocks can thus be propagated at any time of the year and flowering and fruiting problems do not usually arise.

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التكاثر الدقيق للسدر بواسطة زراعة القمة النامية: ١. تحسين المركبات العضوية وغير العضوية لمحتويات البيئات الغذائية لتضاعف المجاميع الخضرية

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المستخلص. شجرة السدر من الأشجار الهامة والقديمة في المملكة العربية السعودية، وهى خلطية التلقيح بطبيعتها، وذات مدى واسع من الاختلافات الوراثية بين أشجارها. وهدف هذه الدراسة هو محاولة إنتاج عدد كبير من الأشجار لنفس التركيب الوراثي المنتخب، والذي يمتاز بجودة الثمار من حيث الطعم، والرائحة، والحجم، والمحصول العالي. فتمت زراعة القمم النامية بطول (١-٠,٥ سم) على عدة بيئات غذائية مختلفة في المحتويات العضوية وغير العضوية، بهدف التوصل إلى أنسب بيئة غذائية في محتوياتها العضوية وغير العضوية، لتعطي أكبر عدد ممكن من المجاميع الخضرية. وأشارت النتائج المتحصل عليها بأن البيئة الغذائية كاملة التركيز من المحتويات العضوية وغير العضوية لبيئة موراشيجى واسكوج (MS) أنتجت أفضل النتائج في طول المجاميع الخضرية، وعدد الأفرع. كما أوضحت النتائج إن طول المجاميع الخضرية، وعدد المجاميع الخضرية قد ازداد زيادة معنوية نتيجة زيادة نسبة السكروز إلى ٣٠ جم/لتر. كما أوضحت النتائج بإن الإينستول لم يكن له تأثير معنوي على طول المجاميع الخضرية

عند مقارنة البيئات الغذائية المحتوية على ١٠٠ مجم إينستول/لتـــر مع البيئات غير المحتوية على الإينستول. كما أوضحت النتائج بأن الفحم النشط قد سبب تحسيناً في طول المجاميع الخضرية، وعددها، وعدد الأوراق، ولم يكن هناك فرق بين استعمال ١ جم/لتـــر، و٢ جم/لتر. كما استعملت في هذه الدراسة تركيزات مختلفة من سالفات الأدنين لدراسة تأثيرها على طول المجـــاميع الخـــضرية، وعـــدد المجاميع الخضرية، وعدد الأوراق المتكونة، فأوضحت النتائج بأن تركيز ٨٠ جم/لتر كان أفضل تركيز لزيادة تنشيط الصفات المعملية المختلفة.