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Full Length Research Paper

In vitro shoot multiplication of *Ziziphus spina-christi* by shoot tip culture

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***In vitro* shoot multiplications were obtained successfully from shoot tips of *Ziziphus spina-christi* by placing explants into solidified medium (MS medium) supplemented with 0.01 mg/l NAA and 0.1 mg/l BA or 0.1 mg/l IAA and 1.0 mg/l kinetin. It was concluded that lower concentrations of all cytokinin studied were better for lateral bud proliferation and that BA and 2IP were better than kinetin in the production of lateral branches. No growth regulators were required for shoot growth and elongation. The shoots rooted best on MS medium supplemented with 1.0 mg/l IBA. Plantlet survival after transfer to soil was more than 90%. The shoot proliferation method described could be used for the mass clonal propagation of selected genotype cv Noaf variety. The variety is in a great demand due to its attractive fruit characteristics such as flavour, sweetness and fruit yield.**

Key words: Clonal propagation, cidir, shoot tip culture, *Ziziphus spina-christi* (L.) Desf.

INTRODUCTION

Ziziphus spina-christi (L.) Desf., locally known as cidir, 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; Saied et al., 2008). It is considered as one of the most drought-resistant fruit crops adapted to the ecological conditions of Saudia Arabia. In the kingdom of Saudi Arabia, the tree grows wild on a wide range of soil types in the Southern and South-western region and has been abundantly cultivated as an irrigated ornamental 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 losses some of its leaves during the winter and somtimes during summer. It is hard, can adapt and grow in a wide range of temperature from below 0 to 52°C. The trees are inexpensive to grow because no irrigation is needed once the tree is established. Two growth cycles have been determined (Abo-Hassan and EL-Osta, 1983).

Z. spina-christi has been shown to have activity against bacteria and fungi (Shahat et al., 2001) and also other pathogens that are normally quite resistant (Nazif, 2002). All parts of the plant are used by the local Arab people to help maintain a healthy lifestyle (Saied et al., 2008). The plant has also been used for its soothing properties (Adzu et al., 2002). In Saudi Arabia, it is used for the treatment of ulcers, wounds, eye diseases and bronchitis. The Bedouin use it for the treatment of wounds, skin diseases and as an anti-inflammatory. They also use it as a febrifuge and diuretic.

Despite the fact that *Z. 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., 1984). Propagation of plants through shoot tip culture allows recovery of genetically stable and true-to type progeny (Barakat and El-Lakany, 1992; Barakat, 2008).

The proliferation of axillary shoot from cultured shoots apices and nodal segments is greatly influenced by the

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Abbreviations: **BAP,** 6-benzyl amino purine; **IAA,** indole-3-acetic acid; **IBA,** indole-butyric acid; **2IP,** isopentenyl adenine; **NAA,** naphthalene acetic acid.

Table 1. Effect of BA and NAA treatment on *in vitro* traits of *Zizphus spina-christi* shoot tip cultures.

NAA (mg/l)	BA (mg/l)	Shoot length (cm)	Number of nodes	Number of leaves	Shoot formation
0.00	0.0	2.22	5.7	4.5	0.0
0.01	0.1	2.09	5.0	4.0	0.7
0.03	0.3	1.75	4.3	3.1	0.3
0.10	1.0	2.71	3.9	2.8	0.2
0.30	3.0	2.03	4.4	3.3	0.1
1.00	10.0	1.41	3.7	2.6	0.0
L.S.D. $_{0.05}$		0.20	1.0	1.1	0.3

nature of the culture medium used. 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. This paper describes an efficient procedure for the mass propagation of the Noaf variety, *Z. spina-christi and* successful establishment of plantlets in the field. This permits the perpetuation of the unique traits of certain strains. The variety is in great demand due to its attractive fruit characteristics such as flavour, sweetness and fruit yield.

MATERIALS AND METHODS

Plant Material and Culture Conditions

Actively growing shoot apices 4 to 5 cm long were obtained from a selected cidir (*Z. spina-christi* Wild) 5 years old tree growing in the vicinity of King Saud University. This single tree was selected on the basis of its heavy yield and fruit characteristics. The leaves were removed except the 6 or 8 youngest ones enclosing the shoot apex. Shoot tip explants were surface sterilized by immersing in 70% ethanol, for a minute, followed by immersing in 0.1% mercuric chloride for 12 min, then washed with six changes of sterile distilled water. The explants were, aseptically, placed on agar-solidified Murashige and Skoog (MS) (1962) based medium containing 3% sucrose varied in growth regulators combinations. MS media were supplemented with a combination of IAA (Indole-3-acetic acid) and kinetin at concentrations of 0.0, 0.01, 0.03, 0.1 and 0.3 mg/l and 0.0, 0.1, 0.3, 0.1 and 0.3 mg/l, respectively. A combination of naphthalene acetic acid (NAA) and benzyle adenine (BA) were also used at concentrations of 0.0, 0.01, 0.03, 0.1 and 0.3 mg/l and 0.0, 0.1, 0.3, 0.1 and 0.3 mg/l, respectively. Different concentration of Indole-3-butric acid (IBA)were tested(0.0, 0.01, 0.03, 0.1 and 0.3 mg/l). Different concentration of 2IP (isopentenyl adenine)were also tested(0.0, 0.01, 0.03, 0.1 and 0.3 mg/l). All media were adjusted to pH 6.0 at 25℃ with KOH and HCL and were autoclaved for 20 min. at 121℃ (15 PSI nominal steam pressure) after the addition of growth regulators as required. The cultures were incubated in a 16 h light and 8 h dark cycle at 25 ± 2℃ and were illuminated with fluorescent light at 2000 lux.

Shoot induction, multiplication and root induction response

Shoot induction response was recorded and the shoot length (cm), number of nodes, number of leaves and number of axillary shoots formed from shoot tips (shoot formation) were made. Shoots with 4 - 6 leaves were transferred to MS medium with IBA at different concentrations from 0.1 to 10.0 mg/l as a rooting medium. Rooted plantlets were removed from the tubes, agar was washed from roots, then the plantlets were transplanted into smal pots filled with compost and covered with polythene bags. The plants were transferred to green house where they were placed under mist for 2 weeks then to an open shadehouse.

Statistical analysis

Data were, statistically, analyzed in a completely randomized design with ten replicates. Number of axillary shoots, number of nodes and number of leaves were subjected to square root transformation prior to statistical analysis (Steel and Torrie, 1980). The data were analyzed, using SAS program, version 6 (1985).

RESULTS AND DISCUSSION

Micropropagation is the true-to-type propagation of selected cultivar using *in vitro* technique. Several stages are involved in micropropagation, each of which is influenced by an array of physical, nutritional and hormonal factors. Micropropagation only makes sense when adequate starting material is used. Therefore, the choice of stock material cannot be made indiscriminately. For most horticulture crops, the starting material is an elite plant selected for a certain phenotype characteristic.

Micropropagation is, thus, similar to the traditional method of vegetative propagation using cuttings but has the distinct advantage of producing greater number of identical plants in a much shorter time (Barakat, 2008). *In vitro* shoot multiplication had been reported in *Ziziphus mauritiana* Lam. (Mathur et al., 1995; Sudhersan et al., 2001) and somatic embryogenesis in *Ziziphus jujuba* Mill (Mitrofanova et al., 1997). However, there are no published reports on the micropropagation of *Z. spina-christi* CV. Noaf. Hence, this study undertook and developed a protocol for the clonal propagation of this Saudi variety.

Effect of NAA and BA on *in vitro* traits

Statistical analysis of shoot length, number of nodes, number of leaves and shoot number (shoot formation) were highly significantly influenced by different combination of BA and NAA (Table 1). The MS medium supple-

Table 2. Effect of IAA and kinetin treatment on *in vitro* traits of *Zizphus spina-christi* shoot tip cultures.

IAA (mg/l)	Kinetin (mg/l)	Shoot length (cm)	Number of nodes	Number of leaves	Shoot formation
0.00	0.0	3.46	4.0	3.70	0.11
0.01	0.1	3.02	4.4	2.44	0.22
0.03	0.3	0.97	1.5	0.10	0.00
0.10	1.0	1.05	1.7	0.00	0.00
0.3	3.0	1.51	2.5	1.00	0.00
$L.S.D._{0.05}$		0.43	0.3	0.45	0.18

Figure 1. Established shoot in hormonal medium with 0.01 mg/l IAA and different concentration of kinetin.

mented with 0.1 mg/l NAA and 1.0 mg/l BA mg/l showed the greatest potential for shoot length (2.71 cm) and it was significantly superior to all other combinations. Number of nodes varied among different combination of NAA and BA. The combination of 1.0 mg/l NAA and 10.0 mg/l BA gave the significantly lowest average mean of nodes number (3.7nodes/explant). However, the differences between the combination (1.0 mg/l NAA and 10.0 mg/l BA) and combinations (0.03:0.3, 0.1:1.0 and 0.3:3.0 mg/l for NAA: BA, respectively) were not significant (Table1). The response for number of leaves varied according to applied medium (Table 1). The medium supplemented with 1.0 mg/l NAA and 10.0 mg/l BA gave, significantly, the lowest mean value of leaves number (2.6 leaves/explants). However, the differences between the combination (1.0 mg/l NAA and 10.0 mg/l BA) and combinations (0.03:0.3, 0.1:1.0 and 0.3:3.0 mg/l for NAA: BA, respectively) were not significant (Table1). In the present study, axillary branching and adventitious shoot regeneration in nodal segments were initiated in the presence of low concentration of NAA: BA (0.01:0.1 mg/l) in the medium and it was significantly superior to all other combinations (Table 1). This was in agreement with the results reported by George (1996), who indicated that choosing an appropriate low plant growth regulators media enabled the proliferation of enough new buds and shoots. This also was in contrast to the results recorded by Fougat et al. (1997), who indicated that for nodal segments bearing an axillary bud and leaf pieces, bud break and shoot proliferation of *Z. mauritiana* were best on an MS medium supplemented with 0.025 mg/l NAA and 1.0 mg/l BA. When the present results on this species were compared with the previous studies on *Z. mauritiana*, it was seen that each cultivar needs appropriate culture medium, with or without growth regulators

Effect of IAA and kinetin on *in vitro* traits

The variance analysis of the shoot tip cultures showed that the effects of the treatments (different combination of IAA and kinetin) were significant on shoot length, number of nodes, number of leaves and shoot formation (Table 2). The MS medium free of auxin and cytokinin showed the greatest potential for shoot length (3.46 cm) and it was significantly superior to all other combinations. Number of nodes varied among different combination of IAA and kinetin. The combination 0.01 mg/l IAA and 0.1 mg/l kinetin gave the significantly highest average mean of nodes number (4.4 nodes/explant). The response for number of leaves varied according to applied medium (Table 2). No leaves derived from nodes of shoot tip cultures were observed on the medium supplemented with 0.1 mg/l IAA and 1.0 mg/l kinetin. However, the highest mean value of leaves number (3.70 leaves/ explant) was obtained on MS free hormones (Table 2). In the present study, more branching was observed in the control and treatments with low concentrations of IAA and kinetin (Figure 1). The role of auxin and cytokinin in the regulation of growth and development of excised plant tissue grown *in vitro* is well documented in the literature (Skoog and Miller, 1957). The effect of both growth regulators depends on the type of the plant, tissue used, media components and objectives of the study and the

Table 3. Effect of 2IP and IBA treatment on *in vitro* traits of *Zizphus spina-christi* shoot tip cultures.

Conc. (mg/l)	Shoot length (cm)	Number of nodes	Number of leaves	Shoot formation
2IP				
0.0	2.8	3.1	2.1	0.21
5.0	1.8	2.0	1.6	0.50
10.0	1.8	2.6	2.1	0.22
15.0	1.9	2.0	2.1	0.22
20.0	1.9	2.6	1.4	0.20
L.S.D.$_{0.05}$	0.8	0.9	1.2	0.37
IBA				
0.0	4.19	4.5	3.7	-
0.1	3.90	4.7	3.5	-
0.5	3.35	3.4	2.8	-
1.0	3.34	3.4	2.9	-
5.0	2.37	3.0	2.0	-
L.S.D.$_{0.05}$	1.13	1.1	0.9	-

concentration of hormones under test. The naturally occurring hormone IAA in this study showed a clear interaction when used in combination with kinetin. The cidir shoot tips were responsive to lower concentrations of kinetin (0.0 or 0.1 mg/l). Stunted growth with less leaves and shorter internodes were obtained at concentration ranging from 0.3 to 3.0 mg/l kinetin, while at these concentrations of kinetin the effect of higher concentration of IAA (0.1 and 0.3 mg/l) on shoot length and other growth parameters was clear. The lack of lateral branching of cidir shoot tips at higher concentration of kinetin indicated that lower concentrations are favorable for both growth and proliferation of buds. It is evident that both growth regulators counteract each other at higher concentrations while at low concentration or lack of both, the growth response seems to be affected by the endogenous auxin in the bud tissue.

Effect of 2IP and IBA on *in vitro* traits

Statistical analysis of shoot length, number of nodes, number of leaves and shoot formation were highly significantly influenced by different concentration of 2IP and different concentration of IBA (Table 3).

2IP exhibited significant influence on the shoot length and number of nodes. The highest shoot length and number of nodes (2.8 cm and 3.1nodes/explant, respecttively) were observed in the medium free of 2IP. The lowest shoot length (1.8 cm) and number of nodes (2.0nodes/explant) were found in medium containing 5 mg/l 2IP (Table 3). The effects of different concentrations of 2IP on number of leaves and shoot number were found to be significant. The results showed that the highest number of leaves (2.7 leaves/explant) and shoot formation (0.50 shoot/explant) were found on medium containing 15.0 and 5.0 mg/l 2IP, respectively and the lowest were 1.4 leaves/explant and 0.20 shoot/explant on medium containing 20.0 mg/l for both *in vitro* traits, respectively (Table 3). The test of 2IP revealed also a similar response where the growth parameters were better in media lacking 2IP, while proliferation of lateral branches was achieved best at 5.0 mg/l 2IP.

On the other hand, the shoot length, number of nodes and number of leaves were highest on medium free of IBA (4.19 cm, 4.5 nodes/explant and 3. leaves/explant, respectively) and lowest (2.37 cm, 3.0 nodes/explant and 2.0 leaves/explant, respectively) on medium containing 5.0 mg/l IBA (Table 3).

Rooting of shoots

Root formation was induced when elongated shoots (1-2 cm) were transferred to MS based agar medium with 0.5 and 1.0 mg/l IBA. The rooted plantlets (Figure 2) could then be transplanted successfully to soil and established under greenhouse. Media methods reported for *Z. mauritiana* (Mathur et al., 1995; Rathore et al., 1992; Goyal and Arya, 1985) failed to induce roots in this species. In the present experiments using IBA concentrations from 0.1 to 5.0 mg/l, root initiation was observed only in media containing 0.5 and 1.0 mg/l IBA. However, the percentage of adventitious root formation was only 60%.

In conclusion, a micropropagation protocol has been developed from shoot tip explants of cidir, which includes three culture phases: 1) Initiation and multiplication, 2) Shoot growth and elongation and 3) Root formation. The three different phases require three different media. The initiation and multiplication medium contains 0.01 NAA mg/l and 0.1 mg/l BA or 0.1 mg/l IAA and 1.0 mg/l kinetin. It was also concluded that lower concentrations of all cytokinin studied was better for lateral bud proliferation

Figure.2. Root formation on MS media supplemented with 1.0 mg/l IBA.

and that BA and 2IP were better than kinetin in the production of lateral branches. The growth and elongation medium contained no growth regulator and the rooting medium contains 0.5 or 1.0 mg/l IBA. Thus, from a single shoot tip explant it is possible to produce thousands of plantlets within a limited time.

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