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Research Article

In vitro Clonal Propagation of a Multipurpose Tree, *Ziziphus spina-christi* (L.) Desf.

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Abstract: A simple and efficient protocol for the clonal micropropagation of *Ziziphus spina-christi* (L.) Desf., a multipurpose native tree species highly adapted to the harsh environmental conditions of Kuwait, has been established using shoot tips and stem nodal segments as explants. The explants were cultured on Murashige and Skoog (MS) basal medium with and without growth regulators. The nodal segments and shoot tips isolated from the primary cultures were cultured on hormone-free MS media containing 100 mg/l myo-Inositol, 150 mg/l glutamine and 2.5% sucrose for plant growth and elongation. Shoots for multiplication were maintained on MS media with low concentrations of 6-benzylaminopurine (BA) and subcultured every 20 days. However, explants cultured in higher concentrations of cytokinin and auxin induced callus. Shoots transferred to the MS media containing 10 mg/l Indole-3-butric acid (IBA) were rooted. Rooted plantlets were transferred to sterile soil media for acclimatisation and field evaluation.

Key Words: *Ziziphus*, micropropagation, cytokinin, acclimatization

Introduction

Ziziphus spina-christi (L.) Desf., locally known as sidr, 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 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 ecological conditions of Kuwait.

Sidr is cultivated mainly as a dry crop for its nutritious fruits, honey production and landscaping purposes. Flowering and fruiting occur in this species during September-November. The flowers are important for the production of wild bee honey (Gaszanfar, 1994). The winter honey (i.e., nabk honey) collected during November from the flowers of the sidr is in high demand by citizens for its medicinal qualities in addition to its excellent taste and fragrant smell.

Sidr is one of the important fruit crops in the dry parts of tropical Asia and Africa. Its fruit is highly nutritious and rich in vitamin C. The dry fruit (i.e., per 100 g) contains 314 calories, 9.3% H_2O, 4.8% protein, 0.9% fat, 80.6% total carbohydrate, 4.4% ash, 140 mg Ca, 3 mg Fe, 0.04 mg thiamin, 0.13 mg riboflavin, 3.7 mg niacin and 30 mg ascorbic acid (Duke, 1985). It is consumed fresh, dried and candied (Bendre & Kumar, 1973). The dried leaves of this plant have long been used as a hair wash in eastern Arabia (Dickson, 1955). They are also used as an excellent leaf fodder for camels and goats. The bark can be used as a source of tannin, and the hard, heavy, termite-proof wood is used in African carpentry.

The antinociceptive activity of aqueous leaf extracts of this species has been studied (Epfraim et al., 1998). The sidr is said to be anodyne, astringent, demulcent, depurative, emollient, laxative, pectoral, refrigerent, stomachic and tonic. It is also used for toothaches and tumours. The powdered seeds mixed with lemon juice are administered for liver complaints, the flower infusion is used as an eye wash and febrifuge, the boiled bark is used to treat venereal diseases, the cathartic raw root juice is used for arthritis and rheumatism, and the fruits are used for bronchitis, coughs and tuberculosis (Hutchens, 1973). The presence of the anti-tumour oestrogen beta-sitosterol (Perdue & Hartwell, 1976), the alkaloids

amphibine A, E, F, mauritine A, and C, and four saponin glycosides in the plant have been reported (Mahran et al., 1993).

The sidr is a heterozygous outbreeder. The only method for the propagation of this species is through seeds. Among the natural seed populations, several cultivars have been noted for their quality fruits. These selected cultivars cannot be propagated through the seedling method. Hence, we tried to develop an alternative method using plant tissue culture for the clonal propagation of sidr and the details of this study are presented herein.

Materials and Methods

Shoot tip cuttings of *Z. spina-christi* were collected from two different healthy, mature trees, showing fruit colour variation (Fig. 1a), growing in the Salmiya area in Kuwait. Shoot tip cuttings were washed in soap water prior to surface sterilisation. The shoot tips were excised and surface sterilised with 20% commercial Chlorox, solution containing 1.05% sodium hypo-chlorite and a drop of Tween 20 for 15 min. After rinsing in sterile distilled water, the leaves were dipped in 0.1% mercuric chloride solution for 3 min followed by rinsing in sterile distilled water three times. Finally, the plant materials were dipped in 70% ethanol for 1 s and rinsed in sterile distilled water. Shoot tip explants were prepared by removing all the expanded leaves, leaving the shoot meristem with 2-3 leaf primordia.

The explants were initially inoculated in Z0, Z1, Z2, Z3 and Z4 media (Table 1) prepared using Murashige and Skoog (MS) basal salts (Murashige & Skoog, 1962). The pH of the media was adjusted to 5.6 prior to autoclaving. All media were dispensed in 25 x 150 mm Sigma test tubes and autoclaved at 121 ºC for 15 min. The cultures were incubated under 1000 lux light intensity provided by white fluorescent lamps for 16-h photo periods at 25 ± 1 ºC. For each treatment, 40 replicates were made, and the experiment was repeated twice. The explants were subcultured once every 20 d. After 45 d, the shoot tips and nodal segments were isolated from the in vitro shoots developed in Z0 media, and inoculated into media containing different concentrations (i.e., 0.0, 0.001, 0.01, 0.1, 1.0 mg/l) of 6-benzyl aminopurine (BA). Isolated plantlets 4-6 cm in length with 4-5 nodes were planted on MS media containing different concentrations (i.e., 0.1, 10, 100 mg/l) of Indole-3-butric acid (IBA) for the rooting experiment. Rooted plantlets were washed and planted on autoclaved soil mix containing sand, peat moss and humus (1:1:1) for acclimatisation.

Results

Among all the used media, the explants in the Z0 medium were healthy and grew vigorously. Elongation of the shoot tip (Figs. 1c,d) was seen after 14 d in culture. There was no callusing at the cut end of the explants. The shoot tip reached 7 cm within 30 d, and each shoot produced an average of 8 nodes within 45 d. There was no axillary branching in this media. Prolonged culturing in this media showed yellowing of the older leaves and hardened stem after 60 d.

In the Z1 medium, the cut end of the explants produced callus (Fig. 1e), and the shoot tip elongated after 15 d. The average shoot length after 30 d was 6 cm, and each shoot produced 8 nodes. Approximately 80% of the shoots produced axillary branches (Fig. 1f) in this media.

In the Z2, Z3 and Z4 media, shoot tip explants callused completely after 30 d. The calli were yellowish and produced nodules (Fig. 1b). There was no shoot development from these calli in the same medium even after a long time in culture.

The nodal segments and shoot tip explants isolated from the primary cultures, developed into shoots with 6-8 expanded leaves after 45 d when transferred to the Z0 medium (Fig. 1g). The shoots were then transferred to the rooting medium to obtain plantlets (Fig. 1h). For multiplication, the plantlets were cut into several segments, each with a node, and cultured in Z0 medium containing different concentration of BA (Table 2) to get the required number of plantlets. Initially the plantlets multiplied at a rate of eight times, and after several subcultures, the multiplication rate increased to 15 times.

Among the concentration of hormones tested for rooting, 30% of the shoots rooted in media containing 10 mg/l IBA. Immediately after root initiation callus formation was observed at the end of the shoots (Fig. 1h). The callusing stopped when the shoots were transferred to hormone-free MS media, immediately after root initiation. Plantlets were transferred to 15-cm diameter pots (Fig. 1i) and maintained in the greenhouse. About 90% of the plantlets survived after the acclimatisation process.

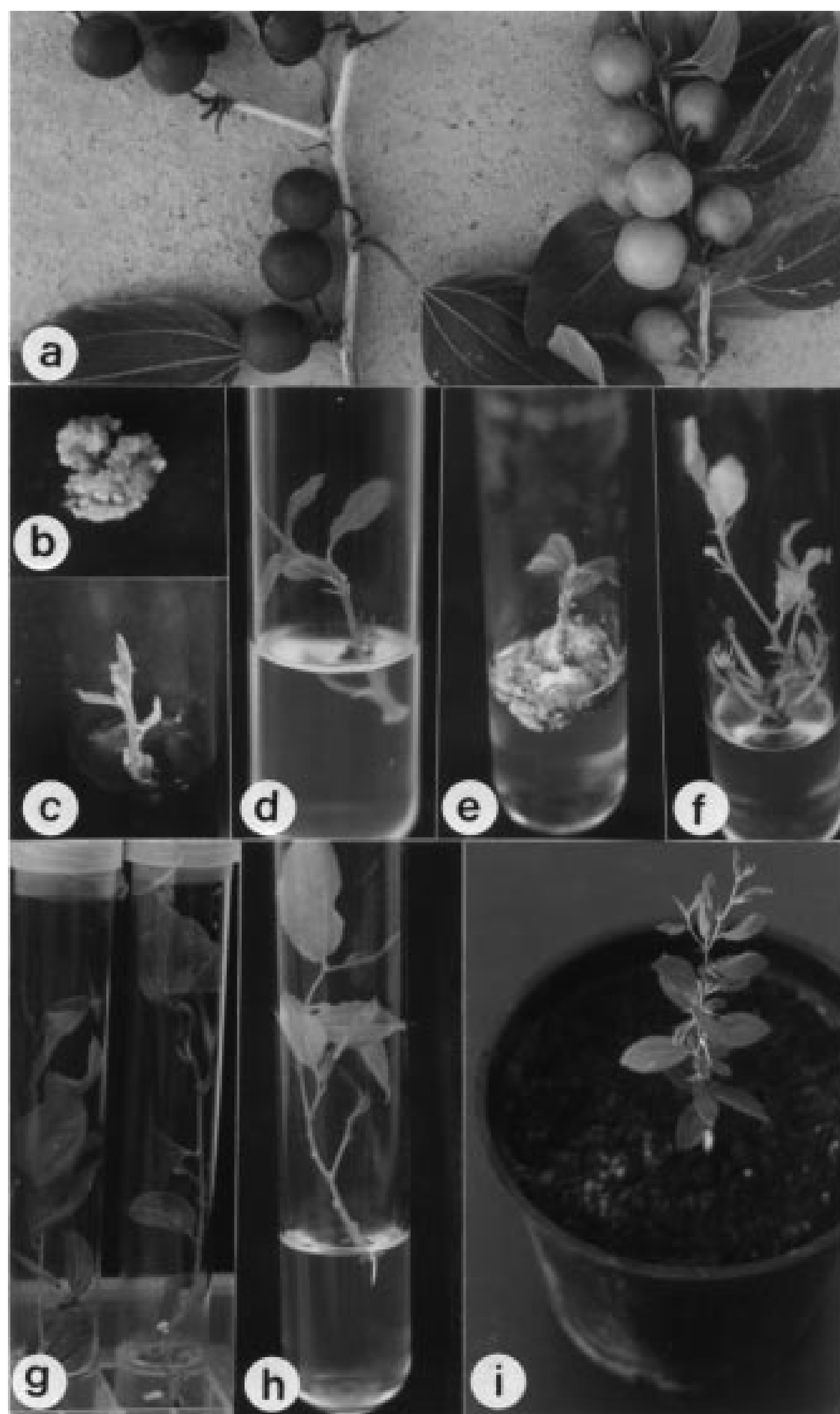

Figure 1. Micropropagation of *Ziziphus spina-christi*. a. Fruiting branches from two different trees showing fruit colour variation; b. Callus formation with nodular structure on Z4 media after 30 d; c. Shoot tip in culture on Z0 media; d. Shoot tip elongation on Z0 media after 14 d; e. Callus formation at the end of shoot tip explants on Z1 media after 14 d; f. Production of axillary branches on Z1 media after 21 d; g. Elongated shoots with 6-8 leaves after 45 d on Z0 media; h. Root formation on MS media supplemented with 10 mg/l IBA after 20 d; i. Acclimatised plantlet in greenhouse.

Table 1. In vitro responses of *Ziziphus spina-christi* shoot tips to MS media with different combinations of plant growth hormones.

Media Code	Media + Hormone (mg/l)	Growth Response
Z0	MS Basal	Growth and elongation
Z1	MS + 1 BA	Growth and multiplication
Z2	MS + 5 BA	Callus formation
Z3	MS + 1 2,4-D	Callus formation
Z4	MS + 5 2,4-D	Callus formation
Z5	MS + 5 2,4-D + 1 BA	Embryogenic callus

Table 2. Effect of MS media with different concentrations of BA on *Ziziphus spina-christi* shoot tip explants after 30 d.

BA Conc. (mg/l)	Mean shoot length	% of branching	Number of nodes
0	7 ± 1	0	8
0.01	6.8 ± 1.7	6	8
0.1	5 ± 1.9	22	7
1.0	3.8 ± 2.2	82	5

(±) Standard error; data from 40 replicates; experiment was repeated twice.

Discussion

It is evident from the results that *Z. spina-christi* can be clonally mass propagated in vitro using shoot tip and nodal segments as explants. Multiple shoot regeneration has been reported in *Ziziphus mauritiana* Lam. (Goyal & Arya, 1985; Mathur et al., 1993; Mathur et al., 1995; Sudhersan et al., 2001) and somatic embryogenesis in *Ziziphus jujuba* Mill. (Mitrofanova et al., 1994, 1997). However, there are no published reports on the micropropagation of *Z. spina-christi*. Hence, we undertook this study and developed a protocol for the clonal micropropagation of this species.

Among the different combinations of MS media used, media without any growth hormones enhanced shoot growth and elongation. Media with auxin or cytokinin induced callusing. However, cytokinins at low concentrations (Table 2) enhanced axillary branching in shoot tip explants and multiple shoot development in nodal segments. The shoot length of the plantlets reached 7 cm in the control, and decreased from 6.8 to 3.8 cm when the BA concentration in the medium increased from 0.01 to 1 mg/l after 30 d (Table 2). The branching percentage of plantlets increased when the BA concentration increased from 0.1 to 1 mg/l. However, more nodes were observed in the control and treatments with low concentrations of BA. When the present results on this species are compared with the previous studies on *Z. mauritiana*, it is seen that each cultivar needs a separate culture medium, with or without growth regulators.

In the present study, axillary branching and adventitious shoot regeneration in nodal segments were initiated only in the presence of low concentrations of cytokinin in the media. Shoot tip explants collected during different seasons responded differently in the cultures. Shoot tips collected in the spring season grew and elongated into plantlets within 30 d on MS hormone-free media, while those collected during summer failed to grow and develop into plantlets on the same media. Shoot tips collected during summer produced plantlets with small scale-leaves after several subcultures in media containing 1 mg/l BA. This seasonal effect on explants may be due to their levels of endogenous growth regulators and their activity.

Media methods reported for *Z. mauritiana* (Mathur et al., 1995; Rathore et al., 1992; Goyal & Arya, 1985) failed to induce roots in this species. In our experiments using IBA concentrations from 0.1 to 100 mg/l, root initiation was observed only in media containing 10 mg/l IBA. However, the percentage of adventitious root formation was only 30%. Moreover, the roots initiated in this media started to callus within a week's time, and affected the root elongation and branching. Transfer to hormone-free media immediately after root initiation controlled the callusing and promoted root growth and branching. Further experiments are being conducted in

our laboratory to obtain a higher percentage of rooting and to refine the rooting technique for this recalcitrant species.

In conclusion, we have developed a micropropagation protocol 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 0.1-1 mg/l BA, the growth and elongation medium contains no growth regulator, and the rooting medium contains 10 mg/l IBA. By the end of three subcultures, about 300 explants were obtained from a single shoot tip, and when these were transferred to the growth hormone-free medium, they gave rise to plantlets measuring 7-8 cm with 6-7 broad and expanded leaves. Thus, from a single shoot tip explant it is possible to produce thousands of plantlets within a limited time.

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