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MICROPROPAGATION OF *Ziziphus jujuba* Mill. (JUJUBE) THROUGH SHOOT TIP AND NODAL SEGMENT CULTURE

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ABSTRACT

Jujube is known as *Masan* in Sri Lanka is one of the underutilized fruit crops. It is important due to its high nutritious value and introduced as a potential crop for commercial cultivation. Micropropagation using plant tissue culture is an efficient method for vegetative propagation of commercially important crops for mass production to meet the demands of planting materials. Present study was carried out to develop a proper *in-vitro* protocol for local varieties of Jujube to produce planting materials. The most suitable fungicide for surface sterilization procedure was selected by testing Captan (Captan 50%, 1.2 g/l), Topsin (Thiophanate methyl 70%, 2 g/l), and Daconil (Chlorothalonil, 1.8 ml/l). Selection of a suitable concentration of BAP (Benzyle Amino Purine) or TDZ (Thidiazuron) for shoot proliferation was assessed in four different concentrations of BAP (1, 1.5, 2, 2.5 mg/l) and of TDZ (0.1, 0.2, 0.3, 0.4 mg/l) and IBA (Indole Butric Acid) for root induction in two concentrations of IBA (1, 2 mg/l). Dipping shoot tips in Captan solution for 20 minutes gave the highest significant non-contamination percentage (79.9%) and lowest fungal radius (0.15 cm) of contaminated cultures among three treatments. MS (Murrashige and Skoog) medium containing 1.5 mg/l BAP recorded significantly highest percentage of elongated bud (96.66%) and newly produced shoot length (1.08 cm) and lowest significant rate was recorded in TDZ 0.2 mg/l (3.33%). Callus was produced in all the concentrations of TDZ. None of the concentrations of BAP or TDZ produced multiple shoots. Elongated nodal segments in BAP (1, 1.5 mg/l) could be successfully sub-cultured for further multiplication. Rooting was not recorded in both shoot tips and *in-vitro* generated shoots during four weeks of culturing on IBA contained media.

***Key words*:** *Ziziphus jujuba* Mill., *in-vitro* propagation, shoot tips, direct shoot induction

INTRODUCTION

Jujube (*Ziziphus jujuba* Mill.) commonly known as *Masan* in Sri Lanka is a perennial fruit tree belongs to genus *Ziziphus* of family Rhamnaceae. Fruits of Jujube are becoming important due to its high nutritious value. Jujube contains good amount of vitamin C and B complexes (Pareek, 2002), minerals (San *et al*., 2009), high amount of antioxidant content (Johnstone, 2012) and excellent source of ascorbic acid and carotenoids (Abbas, 1997). Due to the economic importance, Jujube is commercially cultivated in several countries including China, India, Korea, Japan and Mediterranean countries (Azam-Ali, 2006).

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Jujube is categorized as underutilized fruit crop in Sri Lanka and introduced as a potential crop for commercial cultivation by the Department of Agriculture (Ketipeaarachchi, 2015). In order to promote the cultivation, various propagation methods have been investigated. Jujube can be propagated through seeds and by vegetative methods. Seed propagation is not generally use for fruit production due to its heterozygous nature (Azam-Ali, 2006). Although it is possible to multiply through budding and grafting techniques, the rate of multiplication is very low. Thus to meet the demand of the planting materials for commercialization, it is necessary to obtain a true to type plants through a method of rapid propa-

gation. Micropropagation using plant tissue culture techniques is an efficient method of vegetative propagation of various perennial trees. The tissue culture of *Z. jujuba* Mill. was successfully obtained for several distinguished cultivars *via* leaf (Li *et al*., 2002; Gu *et al*., 2005; Chen *et al*., 2008; Feng *et al*., 2010; Ma *et al*., 2012; Ye *et al*., 2012), embryos (Liu and Qi, 2003, Kim *et al*., 2006), cotyledons (Kim *et al*., 2006) and nodal ex-plants (Goyal *et al*., 2006; Soliman and Hegazi, 2013). However, the response of the planting material on tissue culture media depends on the genotype and therefore, it is important to develop a proper *in-vitro* protocol for Jujube varieties adapted to Sri Lankan condition to promote the commercial scale cultivation. Therefore the present study was carried out with the objectives of determining the most suitable fungicide treatment in surface sterilization procedure, to determine a suitable concentration of BAP or TDZ for shoot induction and to determine a suitable concentration of IBA for root induction of local varieties of Jujube.

MATERIALS AND METHOD

The study was carried out in the Plant Tissue Culture Laboratory at Fruit Research and Development Institute Horana, Sri Lanka during July 2016 to December 2016.

Planting materials

Nodal segments (1.5 cm length) of Jujube were obtained from a healthy, fruit yielding mature trees growing in the conservation garden of Fruit Research and Development Institute, Horana, Sri Lanka.

Surface sterilization

Collected ex-plants were washed under running tap water with few drops of Teepol for one hour. Then they were dipped in three fungicide solutions Captan (1.2 g/l), Topsin (2 g/l) and Daconil (1.8 ml/l) for 20 minutes in each solution separately. Then the ex-plants were washed out with doubled distilled water and shook in 20% Chlorex solution for 15 minutes followed by three rinses of doubled distilled water at the laminar air flow cabinet.

Culture medium and conditions

Ex-plants were cultured on MS basal medium (Murashige and Skoog, 1962) gelled with 7.8 g/l Bacteriological agar. The pH of the medium was adjusted to 5.8 using 0.1M NaOH prior to autoclaving at a pressure of 1.05 $kgcm^{-2}$ and 121^0C for 20 minutes. All cultures were incubated in a culture room at 26 ± 2°C with 3000 lux light intensity provided by cool white fluorescent lamps for 16 hr photoperiod.

Selection of most suitable fungicide treatment

Surface sterilized nodal segments were trimmed into one cm segments and they were cultured vertically on MS basal medium separately according to three fungicide treatments for three weeks to determine the most suitable fungicide treatment in surface sterilization protocol. Fungal radius of the contaminated cultures was measured at four day interval for 20 days.

Culture establishment

The fungicide treatment which gave the highest non-contaminated cultures was selected as the surface sterilization for the shoot tips and nodal segments before culturing them on MS basal medium fortified with four different concentrations of BAP (1, 1.5, 2, 2.5 mg/l) and TDZ (0.1, 0.2, 0.3, 0.4 mg/l) and incubated for four weeks. The percentages of ex-plants initiated growth, percentages of ex-plants produced new shoots, average number of shoots per ex-plant and shoot length (cm) were measured after four weeks of culture.

Shoot multiplication

In-vitro nodal segments obtained from cultures of MS basal medium fortified with BAP at 1 and 1.5 mg/l concentration were sub cultured on same medium for further multiplication. The percentages of ex-plants produced new shoots, average number of shoots per ex-plant and shoot length (cm) were measured after four weeks of culture.

Root induction

Nodal segments and *in-vitro* shoots were cultured on MS basal medium supplemented with IBA at 1 and 2 mg/l concentrations and incubated for four weeks. Root initiation was observed.

Statistical Design and Analysis of Data

Analysis of variance (ANOVA) and Duncan's multiple range test for mean separation were performed to analyze the data using statistical analysis software; SAS institute inc., (2000). Ten cultures were raised for each treatment with three ex-plants per each culture vessel. Experiment was carried out according to Randomized Complete Block Design to test the effectiveness of three fungicide treatments and shoot induction, shoot multiplication and root induction experiments were carried out according to Completely Randomized Design. The differences among averages of the recorded parameters for all treatments were tested for significance at 5% level.

RESULTS AND DISCUSSION

Effectiveness of fungicide treatment in surface sterilization of ex-plant

Dipping ex-plants in Captan (Captan 50%) fungicide solution for 20 minutes which produced 79.9% cultures free of contamination after 20 days of culturing was the most effective fungicide treatment for surface sterilization of nodal segments (Table 4.1).

Table 4.1: Non-contamination percentages of cultures in three different fungicide treatments Topsin, Daconil and Captan used for surface sterilization

Evaluated day after culturing	Topsin	Daconil	Captan
4th day	96.6[a]	76.4[b]	100[a]
8th day	83.2[a]	33.1[b]	93.2[a]
12th day	63.1[a]	16.6[b]	93.2[a]
16th day	46.6[b]	16.6[b]	86.5[a]
20th day	46.6[b]	13.3[b]	79.9[a]

The same letters within the same row are not significantly different at $P \leq 0.05$

Lowest average fungal spreading of contaminated cultures with respect to fungal radius (0.15 cm) was also low in treatment using Captan after 20 days of culture (Table 4.2).

Table 4.2: Growth rate of fungus after contamination calculated as average fungal radius (cm) in the tissue culture medium treated with Topsin, Daconil, Captan.

Evaluated day after culturing	Topsin	Daconil	Captan
4th day	0.0083[b]	0.1583[a]	0.0000[b]
8th day	0.0917[b]	0.5167[a]	0.0330[b]
12th day	0.2583[b]	0.6250[a]	0.0583[b]
16th day	0.4580[ab]	0.6583[a]	0.0833[b]
20th day	0.4083[ab]	0.7083[a]	0.1500[b]

The same letters within the same row are not significantly different at $P \leq 0.05$

Culture establishment

MS basal medium supplemented with various cytokinins (BAP and TDZ) at different concentrations showed that shoot tips and nodal segments of studied cultivar of Jujube could initiate growth on all tested media. The use of MS medium is recommended according to Goyal *et al*. (2006) who reported that for the micropropagation of *Ziziphus jujuba* using nodal ex-plants, MS basal medium was found to be the best among different tested media (B5 and N6) compositions.

After two weeks of culture, bud initiation was observed in the shoot tips and nodal segment ex-plants in MS basal medium with or without any plant hormone (Figure 1 A). Data obtained after two weeks of culture revealed that the percentage of ex-plants initiated bud was significantly affected by the type of hormone as it was not significantly different in control and BAP treatments and significantly different with TDZ treatments. Among the tested concentrations, MS basal medium supplemented with BAP at a concentration of 1 mg/l gave the highest percentage of initiated bud which is not significantly different compared to the other BAP concentrations and the control, for

both shoot tips and nodal segment ex-plants (Table 4.3).

According to the data obtained after four weeks of culture, the percentage of elongated buds was significantly affected by the type of plant hormone and the concentration. Highest percentage of elongated buds (96.66%) was obtained in the MS basal medium supplemented with BAP at a concentration of 1.5 mg/l while lowest percentage (3.33%) was recorded for the treatment using TDZ at a concentration of 0.2 mg/l (Table 4.3). There was a significant difference between the control and BAP treatments in shoot elongation (Figure 1B). Such effects due to known effects of cytokinin in promoting axillary shoot production and its role in plant morphogenesis (Hopkins and Muner, 2008).

No shoot proliferation was recorded in all the tested concentrations of both BAP and TDZ. There was no significant different in control and BAP treatments with respect to the length of shoots of number of ex-plants produced shoots. However it was significantly different in TDZ treatments where the lowest shoot lengths (0.4 and 0.5 cm) were recorded in ex-plants cultured on MS basal medium supplemented with TDZ at 0.1 and 0.2 mg/l concentrations respectively (Table 4.3). This may be due to the one of the undesirable side effect of TDZ is that cultures of some woody species including *Ziziphus jujuba* occasionally form stunted shoots (Lu, 1993).

Shoot tip and nodal segment ex-plants showed callusing in all treatments of TDZ at varying percentages. Initially callus developed at the cut end and latter on entire stem and leaf primodia were callused (Figure 1 C). Callus growth and proliferation was higher in media containing TDZ at 0.4 mg/l concentration (Table 4.4).

Table 4.4: Effect of TDZ concentration on shoot initiation of *Z. jujuba*

TDZ concentration	% ex-plants (callus induction)
Control	0.00^{d}
0.1 mg/l	40.00^{c}
0.2 mg/l	33.33^{c}
0.3 mg/l	70.00^{b}
0.4 mg/l	93.33^{a}

The same letters within the same row are not significantly different at $P \leq 0.05$

Similar results were obtained in the study of Sudhersan *et al*. (2000) on *Ziziphus mauritiana* cv. Umran. However in contrast to results obtained in the present study Soliman and Hegazi, (2013) reported shoot initiation from more than 80% of ex-plants *Ziziphus jujuba*

Table 4.3: Effect of MS basal medium and hormones on *in-vitro* establishment of nodal segments of Jujube

Hormone concentration mg/l	% of ex-plants initiated buds by the 14^{th} day	% of elongated buds by 28^{th} day	Average shoot length of newly produced buds (cm)	Average number of shoots per ex-plant
Control	90^{a}	23.33^{c}	0.95^{ab}	1.0^{a}
1 mg/l BAP	100^{a}	93.33^{a}	0.99^{ab}	1.0^{a}
1.5 mg/l BAP	96.67^{a}	96.66^{a}	1.08^{a}	1.0^{a}
2 mg/l BAP	93.33^{a}	43.32^{b}	0.87^{ab}	1.0^{a}
2.5 mg/l BAP	90.00^{a}	19.99^{cd}	0.8^{b}	1.0^{a}
0.1 mg/l TDZ	34.23^{b}	6.66^{de}	0.4^{c}	1.0^{a}
0.2 mg/l TDZ	33.26^{b}	3.33^{e}	0.5^{c}	1.0^{a}
0.3 mg/l TDZ	33.33^{b}	0.00^{e}	0.0	0.0
0.4 mg/l TDZ	33.33^{b}	0.00^{e}	0.0	0.0

The same letters within the same row are not significantly different at $P \leq 0.05$

Mill. in TDZ at higher concentrations (0.5 mg/l, 1 mg/l and 2 mg/l). Generally the composition of the basal medium, the category and the concentration of plant hormones are the key factors influencing adventitious shoot induction (Feng *et al*., 2010). According to the results of the present study BAP induced the shoot induction of Jujube at lower concentrations and the media containing TDZ suppressed the shoot growth and induced callusing. TDZ has been reported as most active cytokinin like substance to induce adventitious shoot formation in a number of species, especially woody plants (Lu, 1993) including *Z. jujuba* (Gu and Zhang, 2005). However, at concentrations higher than 1 µM, TDZ can stimulate the formation of callus, adventitious shoots or somatic embryos (Lu, 1993).

Shoot multiplication

The nodal segments of in-*vitro* plantlets when subcultured on the MS basal medium supplemented with BAP at 1 mg/l and 1.5 mg/l concentrations, produced shoot bud from each node. No elongation of nodal segments and shoot proliferation were observed (Figure 1 D). Survival percentage and growth percentage reached to 100% on tested treatments. These *in-vitro* nodal segments responded as fresh ex-plants on the medium when subcultured and produced shoots without dwarfing with respect to the length of the shoots (Table 4.5). No multiplication of shoots was observed within 30 days of culture.

Table 4.5: Effect of BAP concentration on shoot multiplication on *in-vitro* nodal segments of subculturing

Cytokinin concentration	% of shooted ex-plants after 28 days of culture	Average shoot length (cm)
Control	13.33[b]	0.87[b]
1 mg/l BAP to 1 mg/	80.00[a]	1.17[a]
1.5 mg/l BAP to 1.5	93.33[a]	1.18[a]

The same letters within the same row are not significantly different at $P \leq 0.05$

Shoot multiplication of several distinguished cultivars of *Ziziphus jujuba* was successfully achieved with MS basal medium supplemented with BAP at 1 and 1.5 mg/l concentrations. Chinese Jujube Dongzao regenerated shoots were grown and proliferated on MS medium containing 1 mg/l BAP with multiplication coefficient of 3.2 (Wang *et al*., 2010). Multiplication of *Z. jujuba* cv. Comethry was achieved with BAP at 1, 1.5 and 2 mg/l concentrations with an average of 3.13 shoots per ex-plant (Soliman and Hegazi, 2013). Multiplication of the studied cultivar in this experiment may be achieved with BAP in long term with regular subculturing as in some cultivars of Jujube, higher rate of shoot multiplication was obtained in long term subculturing. One hundred and six shoots per single shoot tip obtained after six months of culture of *Z. jujuba* cv. Huizao (Abbas *et al*., 2014).

Root Induction

No rooting response was obtained for both shoots and *in-vitro* shoots when cultured on MS basal medium containing IBA at 1 and 2 mg/l concentrations within the period of four weeks. These results were in agreement with the cultivar Zaytoni of *Z. jujuba* reported by Abbas *et al*., (2014) and with the results of cultivar Umran of *Z. mauritiana* reported by Sudhersan *et al*. (2000) where no adventitious root induction occurred at even higher concentrations of IBA on MS basal medium. However 79% of rooting rate was obtained on MS basal medium in cultivar Comethry of *Z. jujuba* Mill. at 2 mg/l IBA concentration after eight weeks of culture (Soliman and Hegazi, 2013).

CONCLUSION

The most effective fungicide treatment was the Captan (Captan 50%) at 1.2 g/l concentration in the surface sterilization procedure of Jujube shoot tips and nodal segments out of other two tested fungicides Topsin (Thiophanate methyl 70%) and Daconil

(Chlorothalonil) with highest non-contamination percentage (79.9%) and with lowest fungal spreading (0.15 cm). The best medium for *in-vitro* establishment of Jujube was MS basal medium supplemented with BAP at 1.5 mg/l concentration with respect to higher percentage of elongated bud (96.66%) and higher shoot length (1.08 cm) of explants produced shoots. BAP or TDZ failed to produce multiple shoots of Jujube in the tested concentrations in MS basal medium. TDZ at concentrations of 0.1, 0.2, 0.3, 0.4 mg/l can be used for the callus induction in plant regeneration program in future. Elongated shoots in BAP (1, 1.5 mg/l) could be successfully sub-cultured in the same media for further shoot multiplication. Root induction of Jujube cannot be achieved in MS basal medium supplemented with IBA at 1 or 2 mg/l concentrations using shoot tips or *in-vitro* generated explants within four weeks period.

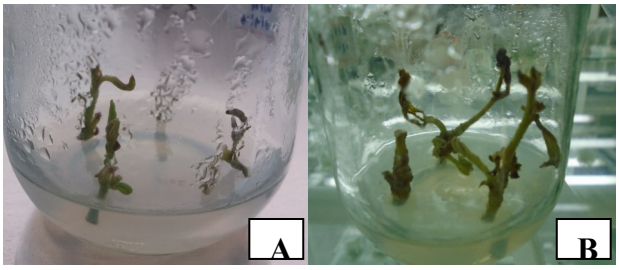

Figure 1: A- Growth initiation of shoot tips cultured on MS basal medium fortified with BAP 1.5 mg/l after two weeks of culture B- Growth and elongation of shoot tips in MS basal medium supplemented with BAP 1.5 mg/l after four weeks of culture

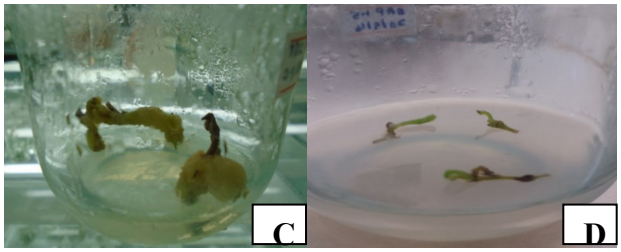

Figure 1: C- Callus growth of shoot tips in MS basal medium supplemented with TDZ after 4 weeks of culture D- Shoot induction of *In vitro* grown segments after two weeks of culture.

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