In Vitro Clonal Propagation and Molecular Characterization of Jujube (*Ziziphus Jujuba* Mill.)

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Abstract: The *in vitro* propagation of three Jujube (*Ziziphus jujuba* Mill.) cultivars was investigated in this study. Nodal segments of *Z. jujuba* were collected from mature plants of Comethry, Balahy and Balady cultivars cultivated in Mariyut Research Station of Desert Research Center, Alexandria, Egypt. The results showed that the best medium for the *in vitro* establishment of Comethry and Balady cv. was Murashige and Skoog (MS) medium containing 0.05 mg l^{-1} β-naphthalene-acetic acid (NAA) + 2 mg l^{-1} N6-(2-isopentenyl) adenine (2iP), but for Balahy cv. this medium was optimum with respect to all parameters, except the growth percentage, which was 100% on MS medium containing 0.05 mg l^{-1} NAA + 1 mg l^{-1} thidiazuron (TDZ). Shoot multiplication rates were significantly affected by the concentration of benzyl adenine (BA), as 4 shoots explant^{-1} were recorded for the Comethry cv. and 4.6 shoots for cultivars Balahy and Balady using MS medium containing 4 mg l^{-1} BA, 4 mg l^{-1} BA + 0.5 mg l^{-1} 2iP and 3 mg l^{-1} BA + 0.5 mg l^{-1} 2iP, respectively. Rooting rates of 78-80% could be produced from shoots cultured on MS medium containing 2 mg l^{-1} indole-3-butyric acid (IBA) for cultivars Comethry and Balahy, and on MS medium containing 2 mg l^{-1} IBA + 0.5 mg l^{-1} NAA for the Balady cv. Rooted plantlets were successfully acclimatized, with 68% survival rate for Comethry cv., 53% for Balahy cv. and 70% for Balady cv., in simple plastic pots containing garden soil, sand and peat moss (1:1:1 v:v:v) in the greenhouse conditions. Genetic diversity among the three studied Jujube cultivars was evaluated using randomly amplified polymorphic DNA (RAPD) and 62.16% of polymorphism was detected between them.

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Abbreviations: **BA**, benzyl adenine; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **2iP**, N6-(2-isopentenyl) adenine; **MS**, Murashige and Skoog; **NAA**, β-naphthalene acetic acid, PGRs, plant growth regulators; **RAPD**, random amplified polymorphic DNA; **TDZ**, thidiazuron.

1. Introduction

Jujube (*Ziziphus jujuba* Mill.) belongs to the Rhamanaceae or bulk thorn family. The genus include about 40 species of spiny shrubs and small trees, distributed in tropical and sub-tropical regions throughout the world. The Chinese jujube (*Z. jujuba*) is a medium-sized perennial fruit tree and medicinal plant, growing 7–10 meters high. The tree is graceful and ornamental in appearance. It has shiny deciduous foliage and produces a fruit with a thin, dark red skin and sweet, white flesh surrounding a stone. Size varies depending on the cultivar and can range from 30 to 120 grams. It has been introduced to more than 30 countries. It is becoming increasingly important for its wide adaptation, easy management, early bearing, rich nutrition and multiple use. The jujube has lower water requirements and higher salt tolerance than most fruits. The tree adapts to drought conditions and not only survives but can produce reasonable yield under severe drought helped by its deep and substantial taproot. The fruit is very nutritious with potassium, phosphorus, calcium and manganese being the major mineral components, as well as iron, sodium, zinc and copper. The jujube is a rich source of vitamin C and vitamin B-complex. The vitamin C content is higher than fruits such as oranges. Studies have shown that the anti-oxidant content of fresh jujubes is higher than strawberries, plums, apples, blueberries, blackberries and raspberries (Johnstone, 2012). Jujube fruits are an excellent source of ascorbic acid and carotenoids (Abbas, 1997). This fruit can be consumed fresh or processed into beverage, food, and oriental medicines.

Jujube flowers can produce high-quality honey, which made up one third of the Chinese honey production in 2007 (Liu, 2008). It has been commonly used as a drug in traditional Chinese medicine as an analeptic, palliative, antibechic and has also been commonly used as food, food additive and flavorant for thousands of years. The Chinese share of world Jujube production is about 90% and its production has increased due to the demands for food and pharmaceutical applications (Li *et al.,* 2005; Su and

Liu, 2005; Li *et al.,* 2009a). Jujube comprises more than 700 cultivars. Molecular genetic methods offer the possibility of making direct and specific changes in existing woody fruit trees while otherwise preserving their integrity (James *et al.,* 1988).

Although, it is possible to multiply Jujube through budding of selected genotypes on seedling rootstocks, the rate of multiplication is very low and therefore is not suitable for mass production of the plant. Thus to meet the demand of planting material, it is necessary to obtain a true to type plants through a method of rapid vegetative propagation. Plant tissue culture is in efficient method of vegetative propagation of various perennial trees. The tissue culture of *Z. jujuba* Mill. is successfully obtained for different distinguished cultivars *via* leaf explants (Chen *et al.,* 2002; Gu and Zhang *et al.,* 2005; Chen *et al.,* 2009; Zhou and Liu, 2009; Feng *et al.,* 2010; QingRong *et al.,* 2011; ChunHua *et al.,* 2012; Ye *et al.,* 2012), embryos (Liu and Qi, 2004; Kim *et al.,* 2006; Wang *et al.,* 2009a), cotyledons (Kim *et al.,* 2006; Wang *et al.,* 2011), nodal explants (Goyal *et al.,* 2006), and others studied the effect of some key factors on the *in vitro* propagation of Chinese Jujube using different explants (MeiQiang *et al.,* 2005; Cao and Du, 2009; Dai *et al.,* 2009).

For the appropriate conservation and utilization of this valuable plant, the genetic diversity between different cultivars should be evaluated. MengJun (2003); MengJun and Jin (2003); Jin and MengJun (2003); Li *et al.* (2009b) and Wang *et al.* (2009b) applied RAPD molecular marker technique for Jujube identification, evaluation of genetic diversity and genetic relationships between cultivars.

In Egypt, this plant is rare, and there are only few cultivars, which needed to be conserved and re-cultured. There are three cultivars in Mariyut Research Station of Desert Research Center, Alexandria, which are named locally as Comethry, Balahy and Balady. The present study aimed to develop an efficient *in vitro* propagation system for rapid and mass propagation of these valuable Jujube cultivars. In addition, RAPD molecular marker technique have been applied for Jujube characterization to improve the use and conservation of this species, and to study the genetic relationships between the three cultivars.

2. Materials and Methods

2.1. In vitro propagation of Jujube

2.1.1. Plant material and sterilization

Nodal segments of *Z. jujuba* were collected from mature plants of three cultivars cultivated in Mariyut Research Station of Desert Research Center, Alexandria, Egypt, namely Comethry, Balahy and Balady (Figure 1). Nodal segments were chosen for the *in vitro* propagation of *Z. jujuba*, because shoot tips were too small and very difficult to be sterilized or survived. The explants were washed under running tap water for 1 hour, followed by a detergent (Pril) for 5 min. Surface sterilization was done by washing the explants with an antioxidant solution (1 g ascorbic acid + 5 g citric acid dissolved in 200 ml sterilized distilled water) under aseptic conditions, then the explants were dipped in 5, 10, 15 and 20% of commercial bleach solution (Clorox containing 1% sodium hypochlorite) for 5, 10 and 15 min, with and without a dittol treatment for 1 min (2 ml dittol in 200 ml sterilized distilled water), and finally explants were rinsed thoroughly with six changes of sterile distilled water. Explants were cut into single node segments and cultured vertically on sterile nutrient medium. Eighty six percent of cultured nodal segments were survived when sterilized with dittol and 20% Clorox for 15 min.

2.1.2. Culture medium and conditions

Explants were cultured on MS basal medium (Duchefa, Haarlem, the Netherlands) (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and gelled with 2.5 g l^{-1} phytagel (Duchefa, Haarlem, the Netherlands). Different plant growth regulators (PGRs) – cytokinins [benzyl adenine (BA), N6-(2-isopentenyl) adenine (2iP) and thidiazuron (TDZ)], and auxins [β-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA)] (Sigma Cell Culture, min. 90%, St. Louis, USA), at different concentrations (mg l^{-1}) – either individually or in combination, were added to the medium to optimize hormonal requirements for bud sprouting, multiple shoot induction and rooting, in addition to the control medium without PGRs. The pH of the medium was adjusted to 5.7-5.8 before autoclaving at a pressure of 1.06 Kg cm^{-2} and 121°C for 15 min. All cultures were incubated in a culture room at 26 ± 2°C with a 16-h photoperiod under cool white fluorescent tubes (F140t9d/38, Toshiba).

2.1.3. Culture establishment and shoot multiplication

Various concentrations and combinations of BA, 2iP or TDZ (0.5, 1, 1.5 and 2 mg l^{-1}) with NAA (0.05 mg l^{-1}) or IAA (0.1 mg l^{-1}) were added to MS medium for the establishment and bud break, in addition to the control medium without PGRs. For multiplication of shoots, MS medium was supplemented with BA at concentrations of 0.5, 1, 2, 3 and 4 mg l^{-1} either singly or in combination with 2iP at 0.5 mg l^{-1}.

The percentage of survived explants, percentage of explants initiating growth, the average number of shoots explant^{-1} and shoot length (cm) were measured after 5 weeks of culture. The

micropropagation cycle consisted of the regular subculture of shoots onto fresh medium.

2.1.4. Rooting and acclimatization of plantlets

The proliferated shoots developed from multiplication stage were singled out from the shoot clusters and cultured on MS basal nutrient medium for 4 week's culture period. Full strength MS medium was used supplemented with various concentrations (1, 2 or 3 mg l^{-1}) of IAA and IBA singly or in combination with 0.5 mg l^{-1} NAA under light conditions with 3 g l^{-1} activated charcoal. Full strength MS medium without PGRs was tested as a control. All cultures were incubated under white fluorescent light at irradiance of 3000 lux in a 16-h photoperiod for 4 weeks. The percentage of rooted shoots, the average number and length (cm) of roots for each rooted shoot were evaluated after 8 weeks of culture on rooting medium.

The rooted plantlets were first transferred to plastic pots containing garden soil, sand and peat moss (1:1:1 v:v:v), covered with transparent polythene bags and placed in acclimatization room at 26±2°C with 70-90% relative humidity, after five days, plantlets were transferred to greenhouse and temperature was increased from 36 to 38°C. After two weeks, transparent bags were gradually removed from pots for proper hardening, and plants were irrigated with tap water every 4 days.

2.1.5. Statistical analysis of data

Analysis of variance (ANOVA) and Duncan's multiple range test (Duncan, 1955), as modified by Snedecor and Cochran (1990), were performed to analyze the obtained data of the *in vitro* propagation of Jujube. At least 15 cultures were raised for each treatment. The differences among averages of the recorded parameters for all treatments were tested for significance at 5% level. Averages followed by the same letter are not significantly different at $P \leq 0.05$.

2.2. Molecular characterization of Jujube

2.2.1. DNA extraction

Genomic DNA was isolated from leaves of Jujube cultivars using the CTAB method, described by Rogers and Bendich (1985).

2.2.2. PCR analysis

PCR reactions were performed in a total volume of 20 µl containing 10 ng DNA, 200 µM dNTPs, 1 µM of 6 arbitrary 10-mer primers (Operon Technology, Inc., Alameda, CA, USA), 0.5 units of Red Hot Taq polymerase (AB gene House, UK) and 10-X Taq polymerase buffer (AB gene House, UK). For DNA amplification Biometra thermal cycler (2720) was programmed at 94º C for 5 min. followed by 35 cycles 94 º C for 1 min., 35 º C for 1 min., 72 ºC for 1 min. and 72 º C for 7 min.

The amplification products were analyzed by electrophoresis in 1% agarose in TAE buffer, stained by ethidium bromide and photographed under UV light. The sequence of the tested primers was as follows: OPE-B-10; CTGCTGGGAC, OPE-C-11; AAAGCTGCGG, OPE-D-08; GTGTGCCCCA, OPE-D-05; TGAGCGGACA, OPE-M-13; GGTGGTCAAG and OPE-N-13; AGCGTCACTC

2.2.3. Band scoring and cluster analysis

The RAPD gel images were scanned using the Gel Doc 2000 Bio-Rad system and analyzed with Quantity One Software v 4.0.1 (Bio-Rad Laboratories, Hercules, Co. USA). The bands were sized and then binary coded by 1 or 0 for their presence or absence in each cultivar. The systat ver.7 computer program was used to calculate the pairwise differences matrix and plot the dendrogram among Jujube cultivars (Yang and Quiros, 1993). Cluster analysis was based on similarity matrices obtained with the unweighed pair-group method (UPGMA) using the arithmetic average to estimate the dendrogram.

3. Results and Discussion

3.1. In vitro propagation of Jujube

3.1.1. Culture establishment

MS basal medium supplemented with various cytokinins (BA, 2iP and TDZ) at different concentrations in addition to NAA or IAA showed that nodal segments of the three studied cultivars of Jujube could survive and form axillary shoots on all the tested media. The use of MS medium is recommended according to Goyal *et al.* (2006) who reported that for the micropropagation of *Z. jujuba* using nodal explants, MS basal medium was found to be the best among different tested media (B5 and N6) compositions.

The survival percentage was high on the tested treatments, for the three cultivars, it ranged between 65 to 100%. Data obtained after 5 weeks of culture revealed that shoots could be induced on all tested media with insignificant difference between tested MS media containing PGRs, with a maximim of 90% for cultivars Comethry and Balady. Although, for Balahy cv., 100% of explants induced growth on MS medium containing 0.05 mg l^{-1} NAA with 1 mg l^{-1} of either BA or TDZ (Table 1 and Figure 2). For the three cultivars, MS medium without PGRs gave significantly the least growth initiation percentage. With respect to the average number of shoots, it was ranged between 1 and 1.2 shoots/explant with insignificant differences between all tested media for the three cultivars. By taking into consideration the shoot length, it was found that it significantly affected by the PGRs combinations and concentrations. Among the tested concentrations, MS medium supplemented with 0.05 mg l^{-1} NAA and 2 mg l^{-1} 2iP produced the highest average length of shoots. This medium produced average lengths of 1.73, 1.93, 1.79

cm for Comethry, Balahy and Balady cultivars, respectively, and it decreased gradually with the decrease in 2iP concentration (Table 1).

Comparing the media containing NAA with those containing IAA, it is obvious that NAA gave better response concerning the average length of shoots, however they were insignificantly different regarding to the other studied growth parameters. With respect to the differences between the used cytokinins, 2iP gave the maximum shoot length if compared to BA or TDZ, singly or if combined with BA. No differences in terms of survival or growth percentage or average number of shoots were observed between the different cytokinins.

Table 1. Effect of MS medium and PGRs (BA, 2iP, TDZ and NAA or IAA) on the *in vitro* establishment of nodal segments of the three cultivars of Jujube (A; Comethry, B; Balahy, C; Balady).

PGRs conc. (mg l^{-1})	% of survived explants			% of explants initiating growth			Average no. of shoots $explant^{-1}$			Average length of shoots (cm)		
	A	B	C	A	B	C	A	B	C	A	B	C
MS control medium (without PGRs)	80^{a}	70^{b}	65^{a}	25^{b}	15^{e}	20^{b}	1.0^{a}	1.0^{a}	1.0^{a}	0.56^{g}	0.50^{g}	0.65^{g}
0.05 mg l^{-1} NAA + 0.5 mg l^{-1} BA	80^{a}	80^{ab}	75^{a}	90^{a}	95^{a}	90^{a}	1.1^{a}	1.0^{a}	1.0^{a}	0.78^{fg}	0.89^{f}	0.81^{fg}
0.05 mg l^{-1} NAA + 1.0 mg l^{-1} BA	75^{a}	85^{ab}	85^{a}	85^{a}	100^{a}	90^{a}	1.0^{a}	1.1^{a}	1.1^{a}	0.86^{ef}	0.79^{f}	0.87^{ef}
0.05 mg l^{-1} NAA + 1.5 mg l^{-1} BA	70^{a}	70^{b}	80^{a}	90^{a}	90^{ab}	80^{a}	1.1^{a}	1.0^{a}	1.1^{a}	0.94^{ef}	0.84^{f}	1.04^{cde}
0.05 mg l^{-1} NAA + 2.0 mg l^{-1} BA	85^{a}	85^{ab}	80^{a}	85^{a}	80^{abc}	80^{a}	1.0^{a}	1.0^{a}	1.1^{a}	1.10^{cde}	1.17^{e}	1.22^{bc}
0.05 mg l^{-1} NAA + 0.5 mg l^{-1} 2iP	80^{a}	85^{ab}	85^{a}	70^{a}	70^{bcd}	80^{a}	1.0^{a}	1.0^{a}	1.0^{a}	1.21^{bcd}	1.29^{de}	1.09^{bcde}
0.05 mg l^{-1} NAA + 1.0 mg l^{-1} 2iP	80^{a}	85^{ab}	80^{a}	65^{a}	65^{cd}	70^{a}	1.0^{a}	1.0^{a}	1.0^{a}	1.68^{a}	1.60^{b}	1.72^{a}
0.05 mg l^{-1} NAA + 2.0 mg l^{-1} 2iP	75^{a}	85^{ab}	80^{a}	75^{a}	55^{d}	75^{a}	1.0^{a}	1.0^{a}	1.0^{a}	1.73^{a}	1.93^{a}	1.79^{a}
0.05 mg l^{-1} NAA + 0.5 mg l^{-1} BA + 0.5 mg l^{-1} 2iP	80^{a}	85^{ab}	85^{a}	80^{a}	75^{abcd}	85a	1.1^{a}	1.1^{a}	1.1^{a}	1.37^{b}	1.46^{bcd}	1.04^{cde}
0.05 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 1 mg l^{-1} 2iP	75^{a}	80^{ab}	80^{a}	80^{a}	75^{abcd}	85a	1.0^{a}	1.1^{a}	1.0^{a}	1.29^{bc}	1.58^{b}	1.20^{bc}
0.05 mg l^{-1} NAA + 0.5 mg l^{-1} TDZ	85^{a}	90^{ab}	85^{a}	80^{a}	75^{abcd}	75^{a}	1.0^{a}	1.0^{a}	1.0^{a}	1.25^{bcd}	1.22^{e}	1.12^{bcd}
0.05 mg l^{-1} NAA + 1.0 mg l^{-1} TDZ	90^{a}	100^{a}	80^{a}	80^{a}	100^{a}	85^{a}	1.1^{a}	1.0^{a}	1.0^{a}	1.09^{cde}	1.49^{bc}	1.30^{b}
0.05 mg l^{-1} NAA + 2.0 mg l^{-1} TDZ	85^{a}	90^{ab}	80^{a}	85^{a}	80^{abc}	90^{a}	1.0^{a}	1.1^{a}	1.0^{a}	1.07^{cde}	1.35^{cde}	1.07^{cde}
0.1 mg l^{-1} IAA + 0.5 mg l^{-1} BA	75^{a}	85^{ab}	85^{a}	75^{a}	80^{abc}	90^{a}	1.0^{a}	1.0^{a}	1.0^{a}	0.84^{ef}	1.3^{cde}	0.88^{ef}
0.1 mg l^{-1} IAA + 1.0 mg l^{-1} BA	80^{a}	85^{ab}	85^{a}	65^{a}	90^{ab}	85a	1.0^{a}	1.2^{a}	1.0^{a}	0.99^{def}	1.44^{bcd}	0.91^{def}

Averages followed by the same letter within a column are insignificantly different at $P \leq 0.05$

In conclusion, the best medium for the establishment of nodal segments of the three studied Jujube cultivars was MS medium containing 0.05 mg l^{-1} NAA + 2 mg l^{-1} 2iP, but for Balahy cv. this medium was optimum with respect to all parameters, except the growth percentage, which was 100% on MS medium containing 0.05 mg l^{-1} NAA + 1 mg l^{-1} TDZ with 1.49 cm average length of shoots (Figure 2). Thidiazuron has been reported to induce adventitious shoot formation in a number of species, especially woody plants (Lu, 1993) including *Z. jujuba* (Gu and Zhang, 2005). In certain species, hyperhydricity of the regenerated shoots has been reported to be associated with using TDZ (Debergh *et al.*, 1992; Feng *et al.*, 2010). Generally, the composition of basal medium, the category and concentration of PGRs, and their combinations are key factors influencing adventitious shoots induction (Feng *et al.*, 2010).

3.1.2. Shoot multiplication

In order to increase the number of shoots $explant^{-1}$, shoots were exposed to 10 treatments of MS basal medium supplemented with BA at 0.5, 1, 2, 3 and 4 mg l^{-1} singly or combined with 2iP (0.5 mg l^{-1}). Data recorded in Table 2 clearly show that explants grown on MS medium supplemented with high concentrations of BA (3 and 4 mg l^{-1}) with or without 2iP gave the highest increase in the average number of shoots, for the three Jujube cultivars.

Shoot multiplication rates were significantly affected by the concentration of BA, as 4 shoots $explant^{-1}$ were recorded for the Comethry cv. and 4.6 shoots for Balahy and Balady cultivars using 4 mg l^{-1} BA, 4 mg l^{-1} BA + 0.5 mg l^{-1} 2iP and 3 mg l^{-1} BA + 0.5 mg l^{-1} 2iP, respectively (Figure 3). Decreasing the concentration of BA significantly decreased the average number of shoots $explant^{-1}$. In this respect, Goyal *et al.* (2006) mentioned that in order to enhance the shoot regenerative potential of *Z. jujuba* shoots, they were cultured on MS medium supplemented with various cytokinins, viz. BA, kinetin and TDZ. MS medium supplemented with BA (1.78 micro M) was found to be ideal for shoot development. In addition, Chinese jujube 'Dongzao' regenerated shoots were grown and proliferated on medium of MS containing 1 mg l^{-1} BA and 0.5 mg l^{-1} IBA with multiplication coefficient of 3.2 (Wang *et al.*, 2010), which is less than the obtained results of the present study.

Table 2. Effect of MS medium and cytokinins (BA and 2iP) on the *in vitro* shoot multiplication of the three Jujube cultivars (A; Comethry, B; Balahy, C; Balady). The survival and growth percentages reached 100% on all tested treatments for the three cultivars.

Cytokinins conc. (mg l^{-1})		Average no. of shoots explant^{-1}			Average length of shoots (cm)		
BA	2iP	A	B	C	A	B	C
0.5	0.0	2.53^{f}	2.53^{e}	3.00^{f}	0.73^{b}	1.05^{a}	0.75^{bc}
1.0	0.0	3.07^{de}	2.87^{de}	3.13^{ef}	0.80^{b}	0.85^{abc}	0.75^{bc}
2.0	0.0	3.20^{d}	3.20^{bcd}	3.20^{def}	0.65^{b}	0.54^{c}	0.70^{c}
3.0	0.0	3.93^{ab}	3.40^{bc}	3.67^{cd}	0.63^{b}	0.58^{bc}	0.68^{c}
4.0	0.0	4.00^{a}	3.60^{b}	4.07^{bc}	0.60^{b}	0.53^{c}	0.61^{c}
0.5	0.5	2.60^{ef}	2.60^{e}	2.53^{g}	2.57^{a}	0.90^{ab}	1.10^{ab}
1.0	0.5	3.13^{d}	3.07^{cd}	3.27^{def}	1.10^{ab}	0.86^{abc}	1.13^{a}
2.0	0.5	3.27^{cd}	3.13^{cd}	3.53^{de}	0.75^{b}	0.76^{abc}	0.86^{abc}
3.0	0.5	3.47^{bcd}	3.47^{bc}	4.60^{a}	0.64^{b}	0.73^{abc}	0.78^{abc}
4.0	0.5	3.73^{abc}	4.60^{a}	4.13^{b}	0.60^{b}	0.67^{bc}	0.76^{bc}

Averages followed by the same letter within a column are insignificantly different at $P \leq 0.05$

The low concentrations of BA and the combination of the two cytokinins; BA and 2iP was more ideal for the shoot length. The longest shoots of Comethry cv. reached 2.57 cm using 0.5 mg l^{-1} BA + 0.5 mg l^{-1} 2iP, and for the Balahy cv. the maximum recorded average shoot length was 1.05 cm on MS medium containing 0.5 mg l^{-1} BA, while for Balady cv., MS medium supplemented with 1 mg l^{-1} BA + 0.5 mg l^{-1} 2iP gave the optimum shoot length, it reached 1.13 cm on this medium.

3.1.3. Rooting and ex vitro acclimatization

From data presented in Table 3 it is clear that IBA was significantly the more efficient auxin type for rooting than IAA. Also, the combination between IBA and NAA gave better rooting response. The highest percentage of explants producing roots (78-80%) was observed on MS medium containing 2 mg l^{-1} IBA for cultivars Comethry and Balahy, and on MS medium containing 2 mg l^{-1} IBA and 0.5 mg l^{-1} NAA for the Balady cv. Also, the maximum average number and length of roots was obtained on these media (Figure 4). No rooting was observed on MS medium without PGRs. In this respect, Chinese jujube 'Dongzao' complete plantlets were obtained after the shoots were rooted on MS medium plus 1 mg l^{-1} IBA with rooting rate of 87.3% (Wang *et al.*, 2010). On the other hand, Gu and Zhang (2005) reported that Nitsch basal medium containing 1.14 lM IAA, 2.46 lM IBA was suitable for rooting of Zhanhua winter Jujube with 90% rooting rate. Also, Feng *et al.* (2010) indicated that 1/2 MS medium supplemented with 2.69 lM NAA was an optimal medium for rooting of Jujube 'Huizao'.

Table 3. Effect of MS medium containing activated charcoal and auxins (IBA, IAA and NAA) on the *in vitro* rooting of the three Jujube cultivars (A; Comethry, B; Balahy, C; Balady).

Auxins conc. (mg l^{-1})			% of explants forming roots			Average number of roots/explant			Average length of roots (cm)		
IBA	NAA	IAA	A	B	C	A	B	C	A	B	C
0.0	0.0	0.0	0^{e}	0^{f}	0^{e}	0.00^{f}	0.00^{f}	0.00^{f}	0.0^{e}	0.0^{e}	0.0^{d}
1.0	0.0	0.0	41^{d}	44^{de}	47^{d}	2.48^{e}	2.68^{d}	1.65^{e}	1.5^{c}	1.2^{d}	1.5^{c}
2.0	0.0	0.0	79^{a}	78^{a}	65^{c}	4.55^{a}	4.55^{a}	2.95^{cd}	2.8^{a}	2.5^{a}	2.3^{ab}
3.0	0.0	0.0	73^{b}	72^{b}	66^{b}	3.58^{c}	3.58^{c}	2.77^{d}	1.4^{c}	1.9^{b}	1.8^{bc}
0.0	0.0	1.0	40^{d}	38^{e}	49^{d}	2.42^{e}	2.42^{e}	2.85^{d}	1.0^{d}	1.2^{d}	1.6^{c}
0.0	0.0	2.0	60^{c}	64^{c}	62^{b}	3.52^{c}	3.52^{c}	3.67^{c}	1.5^{c}	1.8^{bc}	1.4^{c}
1.0	0.5	0.0	64^{c}	66^{c}	52^{cd}	3.11^{d}	3.15^{cd}	3.88^{bc}	2.2^{ab}	2.3^{ab}	2.0^{b}
2.0	0.5	0.0	73^{b}	74^{b}	80^{a}	4.00^{b}	4.00^{b}	4.73^{a}	2.3^{ab}	2.0^{b}	2.9^{a}

Averages followed by the same letter within a column are insignificantly different at $P \leq 0.05$

In the root meristem, auxin is implicated in regulating the pattern of cell division and differentiation (Friml, 2003). According to Puente and Martin (1997), if the shoots are competent to root, rooting rate could be increased easily. It has been reported that shoot characteristics such as size and shoot culture origin fail to attain a stabilized growth phase or apparent rejuvenation can also lead to a variable rooting response (Marks and Simpson, 2000).

Rooted plantlets were successfully acclimatized, with 68% survival rate for Comethry cv., 53% for Balahy cv. and 70% for Balady cv., in simple plastic pots containing garden soil, sand and peat moss (1:1:1 v:v:v) in the greenhouse conditions. They did not show any detectable morphological variation (Figure 5).

To conclude, the outlined procedure offers a potential system for mass propagation of the three Jujube cultivars; Comethry, Balahy and Balady, from nodal explants. Such reproducible protocol could be useful in producing a true-to-type and continuous germplasm of this economically important plant.

Figure 1. Forms of fruits of the three Jujube cultivars, A; Comthery, B; Balahy and C; Balady.

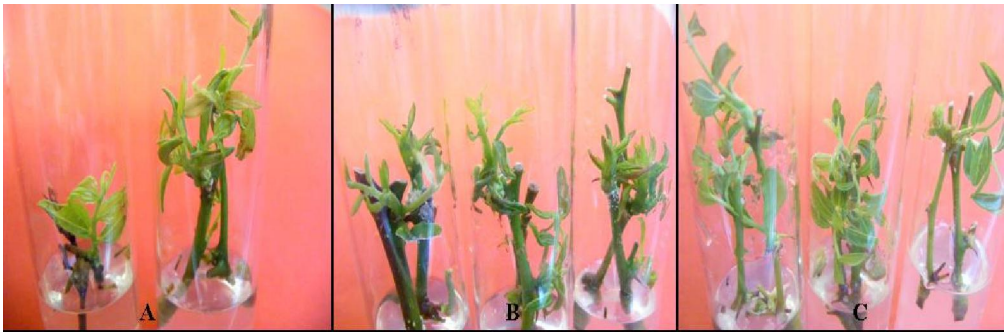

Figure 2. *In vitro* establishment of stem node sections of the three Jujube cultivars, A; Comthery, B; Balahy and C; Balady.

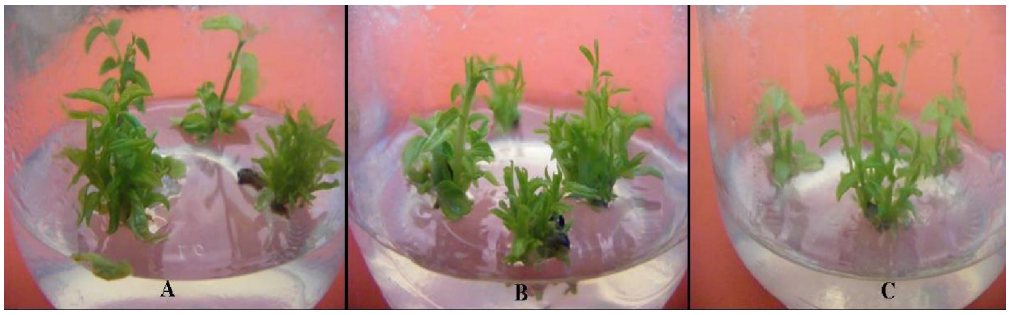

Figure 3. Shoot multiplication of the three Jujube cultivars, A; Comthery, B; Balahy and C; Balady.

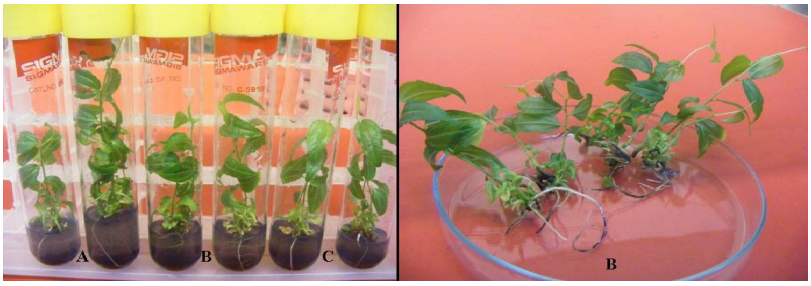

Figure 4. *In vitro* rooted plantlet of the three Jujube cultivars, A; Comthery, B; Balahy and C; Balady.

Figure 5. Acclimatized plantlets of Jujube cultivars, A; 4 weeks after potting and B; 6 months after potting.

3.2. Molecular characterization of Jujube

The RAPD technique was employed to detect the genetic variations between the three Jujube cultivars. Genomic DNA of the three Jujube cultivars were extracted and were used in performing RAPD by six arbitrary oligonucleotide primers (Figure 6). The number of amplified fragments differed from one cultivar to another, which indicating that cultivars are not always identical in their DNA ability to be amplified.

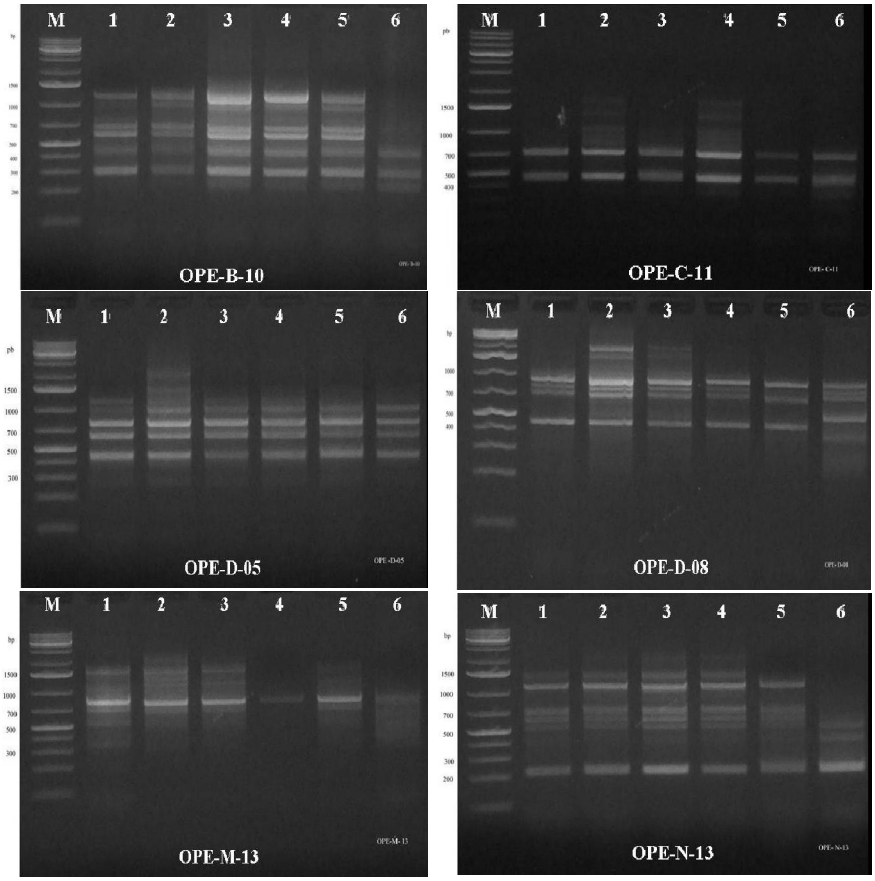

Figure 6. DNA banding pattern of the three Jujube cultivars generated by six primers.
Lane M: DNA marker, lanes 1, 2: bands of Comethry cv., lane 4: bands of Balahy cv., and lanes 5,6: bands of Balady cv.

The total number of scorable bands of each primer varied from 5 to 7. The size of these amplification products range between 200-1500 bp (Table 4). All six used RAPD primers generated polymorphic bands; The OPE-M-13 primer recorded the highest percentage of polymorphism (80%) as it revealed 4 polymorphic bands from

5 amplified fragments, while the OPE-D-05 primer recorded the least percentage (33.33%) by revealing 2 polymorphic bands from 6 amplified fragments. The 6 primers produced 37 bands for all Jujube cultivars. A total of 14 bands (37.84%) were monomorphic, and 23 bands (62.16%) were polymorphic. Similar results were obtained by Peng *et al.* (2000) who analyzed the genetic relationships of 64 Chinese date (*Z. jujuba*) cultivars using RAPD markers, and found 59.55% polymorphism. Also, 79.17% polymorphism was detected by Li *et al.* (2009c) when they aimed to evaluate the genetic structure of *Z. jujuba* 'Huizao'. They suggested collecting some sample trees by the phenotypic variation for germplasm conversation and new cultivar selection.

Table 4. List of primers, their nucleotide sequence and amplification results of the three Jujube cultivars (A; Comethry, B; Balahy, C; Balady).

Primer code	**Sequence (5' ------- 3')**	**Number of amplified fragments**			**Length of amplified fragments**
		A	B	C	
OPE-B-10	5`-CTGCTGGGAC-3`	7	7	7	200-1500bp
OPE-C-11	5`-AAAGCTGCGG-3`	6	6	2	400-1500bp
OPE-D-05	5`-TGAGCGGACA-3`	6	5	5	300-1500bp
OPE-D-08	5`-GTGTGCCCCA-3`	5	4	6	400-1000bp
OPE-M-13	5`- GGTGGTCAAG-3`	5	1	4	300-1500bp
OPE-N-13	5`-AGCGTCACTC -3`	6	6	5	200-1500bp
Total		**35**	**29**	**29**	**93**

Table 5. Polymorphism rate of the three Jujube cultivars using six RAPD primers.

Primer code	**Sequence (5' ----------3')**	**Total amplified fragments**	**Polymorphic fragments**	**Polymorphism (%)**
OPE-B-10	5`-CTGCTGGGAC-3`	7	5	71.43
OPE-C-11	5`-AAAGCTGCGG-3`	6	4	66.67
OPE-D-05	5`-TGAGCGGACA-3`	6	2	33.33
OPE-D-08	5`-GTGTGCCCCA-3`	6	3	50.00
OPE-M-13	5`-GGTGGTCAAG-3`	5	4	80.00
OPE-N-13	5`-AGCGTCACTC -3`	7	5	71.43
Total		**37**	**23**	**62.16**

UPGMA dendrogram obtained from the data of RAPD analysis showing the relation between the three Jujube cultivars is presented in Figure 7. UPGMA cluster analysis generated a dendrogram with two clusters, separating cultivars Comethry and Balahy from Balady cv., which occupied a distinct place and distantly related to the other cultivars. The Comethry cv. was closely related to the Balahy cv., they formed a cluster comprised of the two cultivars.

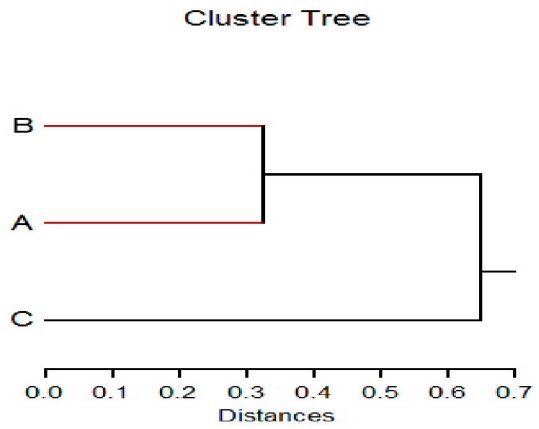

Figure 7. A dendrogram of the three Jujube cultivars developed from RAPD data based on UPGMA analysis, A; Comthery, B; Balahy and C; Balady.

In conclusion, RAPD was informative for estimating the extent of genetic diversity as well as to determine the pattern of genetic relationships between different studied Jujube cultivars with polymorphism levels sufficient to establish informative fingerprints with relatively fewer primer sets. The degree of genetic variation detected in Jujube cultivars with RAPD analysis in the present study suggests that it is an efficient marker technology for delineating genetic relationships among cultivars and estimating genetic diversity, thereby enabling the formulation of appropriate strategy for conservation and improvement programmes. Genetic diversity within species is usually related to geographical range, mode of reproduction, mating system, seed dispersal and fecundity (Gupta *et al.*, 2008 and Subramanyam *et al.*, 2010). The more genetic variations within a species, the greater is its potential to adapt to environmental changes (Rae, 1999).

Collection and conservation of rare and endangered germplasm and identification and evaluation of germplasm with high resistance and nutrition characters should be strengthened and emphasized. Physiology, biochemistry, heredity and breeding techniques should be studied thoroughly. The germplasm management system, the network information platform construction and the germplasm utilization system should be perfected, so as to improve the efficiency of utilization (Wang *et al.*, 2009b).

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