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Influence of Plant Growth Regulators and Medium Strength on Micropropagation of the Biodiesel Plant, *Jatropha curcus*

Mohamed R. Rady, El-Sayed, M. Mervat[1], Mohamed El-Sayed and Usama I. Aly*

Department of Plant Biotechnology, Genetic Engineering and Biotechnology Division, National Research Center, El-Buhouth St., (P.O. Box 12622), Dokki, Cairo, Egypt.

Key words: Jatropha curcas, Medium strength, Micropropagation

Abstract

Leaf explants were cultured to evaluate the effect of different auxins and cytokinins and its concentrations; MS salts on micropropagation of the promising biodiesel *Jatropha curcas* plant under Egyptian conditions. Results showed that shoot initiated on 0.5 mg/l BA + 0.25 mg/l IBA. Multiplication and elongation were found to be the best using 0.5 mg/l BA in combination with 0.05 mg/l IBA. The multiple shoots were cultured on MS or half strength of MS supplemented with different concentrations of IAA and IBA for rooting phase. Half strength of MS containing 1.0 mg/l IAA was the best for rooting of micropropagated shoots. The rooted plantlets were acclimated in sand : peat-moss mixture (1 : 1) successfully.

Introduction

Jatropha curcas L. or physic nut belongs to *Euphorbiaceae* is an all-purpose, zero-waste perennial promising plant. It is considered as a potential source of non-edible biofuel along with its different medicinal properties and many other uses (Datta et al. 2007). The high yield of seed from the tree, the high oil content of seeds and low production cost have attracted global attention for the development of *J. curcas* as a source for biofuel (Li et al. 2007).

Conventionally seeds and cuttings of *J. curcas* are used for its propagation. *Jatropha curcus* is cross-pollinated and as such the seeds are heterozygous with poor viability and low germination while the cuttings are used seasonally (Datta et al. 2007). Moreover, it is reported that vegetative cuttings are not deep-rooted

*Authors for correspondence: <usamajp@yahoo.com>. [1]Department of Chemistry, Faculty of Science, Cairo University, Cairo, Egypt.

as they do not form a taproot system (Sujatha et al. 2005) while seedlings from seeds have a taproot system showing a lower longevity and possess a lower drought and disease resistance and lower seed set than those propagated by seeds (Heller 1996). In addition, plant species with rich secondary metabolites have proved to be difficult for mass propagation through tissue culture (Kalimuthu et al. 2007).

Biodiesel, an alternative diesel fuel, has attracted considerable attention during the past years as a renewable biodegradable and nontoxic fuel. Major sources of biodiesel include rapeseed (USA), sunflower (Italy and Southern France), soybean (USA and Brazil), oil palm (Malaysia), linseed (Spain), cotton seed (Greece), beef tallow (Ireland) and *Jatropha* (Nicaragua and South America) (Jayasingh 2004). *J. curcas* has the potential to become one of the world's key energy crops. The yield of oil from kernels is estimated between 46 and 58% derived from semi-drying oil (Kalimuthu et al. 2007) that contains more oxygen, with a higher cetane value increasing the combustion quality and this reduces the environmental pollution. *Jatropha* oil can be used directly in diesel engines as an extender or transesterized to a biodiesel fuel. It has greater lubricity and reduces engine wear (Datta et al. 2007).

A large number of plants for commercial purpose may be propagated through stem cuttings but the yield of seeds is low and the matured plants are not deep rooted and hence, are easily uprooted. This is a major constraint for establishment of plants propagated through stem cuttings on poor and marginal soils.

Considering its enormous potential, a large amount of quality planting material is required for future use (Datta et al. 2007, Dubey et al. 2010). Thus, improvement of this crop through the application of tissue culture is keenly felt. Moreover, the multiplication cycle is very short; so it is important for large scale production of *J. curcas* plant. So, it is necessary to find out ways and means for over coming the conventional propagation problems and to mitigate the needs from fuel by increasing the area for cultivation (Adebowale and Adedire 2006).

The present investigation has been carried out to optimize a protocol for a large scale production of *J. curcas* plants using plant biotechnology techniques including establishment of protocols for shoot initiation, multiplication, rooting and acclimation of *J. curcas* plants grown in Egypt.

Materials and Methods

Three months old *Jatropa curcas* seedlings were provided by the the nursery of the Ministry of Agriculture, Giza, Egypt. The work was carried out in Plant

Biotechnology Department, Genetic Engineering and Biotechnology Division, National Research Center, Cairo, Egypt.

MS with 30 g/l sucrose was used. Seven g/l agar were added to solidify the medium. The pH of the medium was adjusted 5.6 to 5.8 and were sterilized by autoclaving at 121ºC and 15 psi for 20 min. All cultures were incubated under controlled conditions in the growth chamber. Temperature was maintained at 25 ± 2°C at a photoperiod of 16 hrs light/8 hrs dark with illumination intensity of 2000 lux from cool white fluorescent lamps (120 cm long).

Nodal explants were excised from *J. curcas* seedlings and rinsed in tap water followed by agitating them in a disinfectant solution of Savlon (3%) for 30 min. Thereafter the materials were soaked in 70% ethanol for 1 min under aseptic conditions in a vertical air laminar flow cabinet. Explants were dipped in 0.1% $HgCl_2$ for 5 minutes and then soaked min 1.5% NaOCl for 10 minutes.

Sterilized nodal explants were cultured on MS supplemented with various concentrations of BA and/or IBA. Number of proliferated shoot buds, leaves and shootlets length (cm) were recorded after four weeks of cultivation. Shoots were cultured on MS supplemented with different concentrations of BA and/or IBA.

In vitro shootlets of *J. curcas* were used for root formation using different treatments on half strength of MS and MS with different concentrations of IAA or IBA. Number of days for root initiation, root formation percentage, number of roots and roots length (cm) were recorded after six weeks.

In vitro rooted plantlets were washed with sterile distilled water to remove medium residues and dipped in 0.01% Bavistin solution (fungicide) for 15 min, then transplanted into 0.2 liter capacity pots and filled with three types of sterilized soil mixture (v/v) as follow : sand : peat (1 : 1), sand : peat : perlite (1 : 1 : 1) and sand : peat : vermiculate (1 : 1 : 1) for acclimation. Pots were covered with transparent polyethylene bags and maintained under controlled conditions (16/8 hrs light/dark and 25 ± 2°C). After one week, the cover of polyethylene bags was perforated with one hole for 2 weeks two times. After one month the polyethylene bags were removed followed by their transfer into pots and thereafter to the greenhouse. At the end of acclimation 12 weeks in the greenhouse, survival percentage, the length of plantlets and the number of leaves per seedling were recorded.

Data are expressed as the means of triplicates. The error bars in the charts indicate standard error (S.E.). The least significant difference (LSD) test at 0.05 level probabilities was used to compare mean values of all treatments.

Results and Discussion

Data in Table 1 showed the effect of different combinations of BA and IBA on shootlets proliferation. MS supplemented with 0.5 mg/l BA + 0.25 mg/l IBA (T8) gave the highest number (9.33) of proliferated shoots (Fig. 1A). On the other hand, MS gave the lowest number (0.33) of proliferated shoots (Fig. 1B). A positive response associated with the increase of BA concentrations was observed from 0.0 to 0.5 mg/l BA then decreased with 1.0 mg/l BA. In addition, the highest number of leaves per proliferated shoot (8.33) was observed on the same medium. The lowest number (1.33) was recorded in MS. Moreover, regardless of the concentration of IBA, there was a positive response in shootlet length with the increase of BA up to 1.0 mg/l. Maximum shootlet length (3.67 cm) was recorded with MS supplemented with 0.5 mg/l BA + 0.25 mg/l IBA, whereas the minimum shootlet length (1.57 cm) was observed in MS.

Table 1. The effect of different concentrations of BA and/or IBA on the initiation of *J. curcas* shootlets from nodal explants after one month of culture.

Media code	Growth regulators (mg/l)		No. of proliferated shoot buds	No. of leaves	Shootlet length (cm)
	BA	IBA			
T1	0.0	0.0	0.33f	1.33d	1.57b
T2	0.0	0.25	0.67ef	1.67dc	1.80b
T3	0.0	0.5	0.67ef	1.67dc	1.77b
T4	0.25	0.0	2.67def	5.00b	2.73ba
T5	0.25	0.25	3.67dc	6.00ba	2.97ba
T6	0.25	0.5	3.67dc	6.00ba	3.00ba
T7	0.5	0	6.33bc	7.00ba	3.07ba
T8	0.5	0.25	9.33a	8.33a	3.67a
T9	0.5	0.5	7.33ba	6.67ba	3.03ba
T10	1.0	0.0	3.33de	6.00ba	2.30ba
T11	1.0	0.25	3.33de	6.33ba	1.77b
T12	1.0	0.5	3.00def	4.33bc	1.63b
$LSD_{0.05}$ (Pr> F 0.001)			2.995	2.937	1.666

Within each column, values followed by the same superscript are not significantly different at the p = 0.05 level according to the least significant difference (LSD) test.

In general, it is concluded that MS supplemented with 0.5 mg/l BA + 0.25 mg/l IBA was the best medium for shoot initiation from nodal explants of *J. curcas* as it gave the highest number of proliferated shoots, leaves and the longest shootlet length. In this respect, Sujatha and Dhingra (1993) found that the

presence of BA (0.5 or 1.0 mg/l) and IBA (1.0 mg/l) in MS initiated and proliferated adventitious shoot buds of *J. integerrima*. Rajore and Batra (2005) reported that MS supplemented with 2.0 mg/l BA and 0.5 mg/l IAA in combination with adenine sulfate, glutamine and activated charcoal was the best for proliferation of shoot tip explants of *J. curcas*. Moreover, MS supplemented with 0.5 -1.0 mg/l BA was found to be most effective for shoot induction from apical shoots of *J. curcas*. Moreover, shoot induction from nodal explants required MS supplemented with 1.0 mg/l BA, whereas, the best shoot proliferation from axillary buds was achieved on MS supplemented with 0.5 mg/l BA in combination with 0.01 mg/l IBA (Thepsamran et al. 2008).

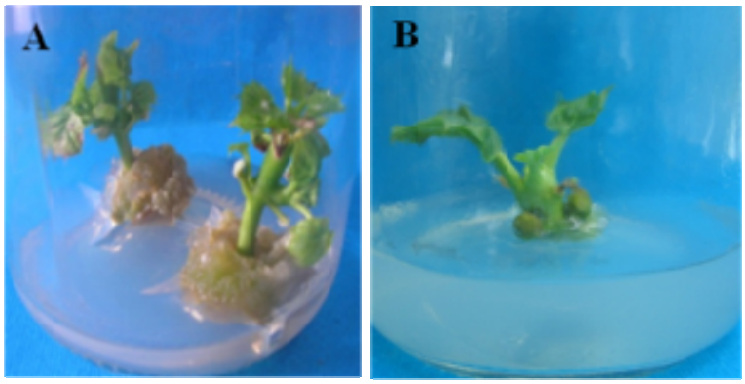

Fig. 1. Shoolets initiation from nodal explants of *J. curcas* cultured on MS supplemented with; A: 0.5 mg/l BA + 0.25 mg/l IBA and B: MS basal medium (Control) after one month of cultivation.

Present results are in agreement with those obtained by Kaewpoo and Te-chato (2009) who found that MS supplemented with 0.5 mg/l BA and 0.25 mg/l IBA was the best for proliferation of stem, axillary bud, and shoot tip explants of *J. curcas* providing the highest number of shoots per responding explant. Also, Mohanalakshmi et al. (2009) mentioned that MS fortified with 1.5 mg/l BA was highly effective giving 76.4% success in axillary buds proliferation in *J. curcas*. Recently, Purkayastha et al. (2010) demonstrated that MS supplemented with 0.77 mg/l BAP was the best media for shoot induction (6.2 shoots per shoot apex) from shoot apices of *J. curcas* plants.

Misra et al. (2010) reported that MS supplemented with 0.5 mg/l BA and 0.1 mg/l IBA was the best for *J. curcas* shoot initiation from nodal explants. Also, Dubey et al. (2010) reported that *in vitro* shoot regeneration from shoot buds explants of *J. curcas* plant was induced on MS supplemented with 1 mg/l BAP in combination with 0.5 mg/l IBA. Moreover, they reported that induced shootlets

were elongated at an average length of 3.77 cm on MS supplemented with 0.5 mg/l BAP in combination with 1.0 mg/l GA_3.

Shootlets were transferred to shoot multiplication media consisting of MS supplemented with different combinations of BA and IBA to study their effects on multiplication of *J. curcas* shoot.

Data in Table 2 showed the effect of different combinations of BA and IBA on multiplication of *J. curcas* shootlets. The highest number of proliferated shoot buds (13.67) was reported in the medium supplemented with 0.5 mg/l BA + 0.05 mg/l IBA (T16) (Table 2), while, the lowest number of multiplied shoots (0.67) was recorded in MS (T1) (Table 2). With regard to number of leaves it was observed that, MS supplemented with 0.5 mg/l BA + 0.05 mg/l IBA (T16) (Fig. 2A) and MS supplemented with 1.0 mg/l BA + 0.1 mg/l IBA (T20) gave the highest number (14.67) of leaves (Table 2). Hence, the lowest number of leaves (3.00) was observed when the explant was grown in MS.

Table 2. Effect of different concentrations of BA and IBA on multiplication of *J. curcas* shootlets derived from the initiation stage after one month of cultivation.

Media codes	Growth regulators (mg/l)		No. of proliferated shoot buds	No. of leaves/proliferated shoot bud	Shootlet length (cm)
	BA	IBA			
T1	0.0	0.0	0.67e	3.00c	2.17e
T13	0.25	0.05	3.67g	8.00b	3.50bc
T14	0.25	0.1	5.67egf	8.67b	3.60bac
T15	0.25	0.2	5.33gf	8.67b	3.90ba
T16	0.5	0.05	13.67a	14.67a	4.20a
T17	0.5	0.1	11.33b	8.67b	4.17a
T18	0.5	0.2	10.33cb	10.33cb	4.23a
T19	1.0	0.05	7.00edf	10.33cb	3.30bdc
T20	1.0	0.1	7.67edf	14.67a	2.27e
T21	1.0	0.2	7.67edf	8.33b	3.50bc
T22	1.5	0.05	8.33cd	9.00b	2.10e
T23	1.5	0.1	7.00edf	7.67b	3.13dc
T24	1.5	0.2	8.00ed	10.00cb	2.70ed
$LSD_{0.05}$ (Pr > F 0.001)			0.95	2.15	0.069

Within each column, values followed by the same superscript are not significantly different at the p = 0.05 level according to the least significant difference (LSD) test.

Concerning the shootlet length it was observed that the maximum shootlet lengths were 4.23 and 4.20 observed in the MS supplemented with 0.5 mg/l BA + 0.20 mg/l IBA (T18) (Table 2) and with 0.5 mg/l BA + 0.05 mg/l IBA (T16) (Table

2), respectively. But the lowest shootlet length (2.10) was observed in the MS supplemented with 1.5 mg/l BA + 0.05 mg/l IBA (Table 2).

Results showed that MS supplemented with 0.5 mg/l BA in combination with 0.05 mg/l IBA was the best for multiplication of *J. curcas* shootlets. Sujatha and Dhingra (1993) showed that MS supplemented with a combination of 0.5 or 1.0 mg/l BA and 1.0 mg/l IBA was the best combination for the development and multiplication of buds of *J. integerrima*. Moreover, Krishna et al. (2010) used MS supplemented with 2.0 mg/l of BAP in combination with 1.5 mg/l IBA for development and multiplication of shootlets of *J. curcas* with high shootlet lengths. *In vitro* regenerated plantlets of *J. curcas* were subcultured in MS supplemented with 0.5 mg/l BA and 0.25 mg/l IBA for multiplication (Kaewpoo and Te-chato 2009). It was also reported that, the best length for shootlets of *J. curcas* was obtained in MS supplemented with 0.3 mg/l BA (Nogueira et al. 2011).

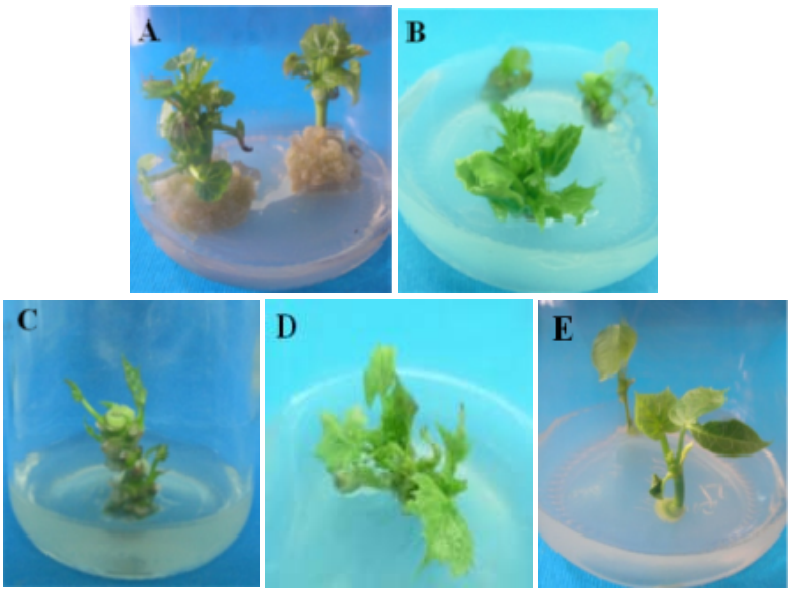

Fig. 2. Shoot multiplication from nodal explants of *J. curcas* on MS supplemented with; A: 0.5 mg/l BA + 0.05 mg/l IBA, B: 1.0 mg/l BA + 0.1 mg/l IBA, C: 0.5 mg/l BA + 0.2 mg/l IBA, D: 1.5 mg/l BA + 0.05 mg/l IBA and E: basal medium, after one month of cultivation.

The shootlets were used for rooting in different treatments. Data represented in Table 3 showed the effect of two different rooting media on root formation and their characteristics in shootlets of *J. curcas*. Half strength of MS and MS with different concentrations of IAA or IBA were used (Table 3). The highest

percentage of root formation (100) occurred in half strength of MS (T35) supplemented with 1.0 mg/l IAA (Table 3), followed by 90% in MS (T25) supplemented with 1.0 mg/l IBA (Table 3) and MS (T27) supplemented with 3.0 mg/l IBA (Table 3), half strength of MS (T37) supplemented with 3.0 mg/l IAA (Table 3) also recorded 90% root formation. On the other hand, MS (full and half strength) MS (T28) supplemented with 1.0 mg/l IAA and half strength MS (T33) supplemented with 2.0 mg/l IBA did not induce any root. Generally, half strength of MS supplemented with 1 mg/l IAA was the best for giving the highest percentage of root formation for *in vitro* produced shootlets of *J. curcas*. In addition, the highest number of roots per shootlet (4.50) was observed in with full MS (T25) supplemented with 1.0 mg/l IBA, whereas, half strength MS (T34) supplemented with 3.0 mg/l IBA gave the lowest number (0.25) of roots per explant. Generally, MS treatments were better than half strength of MS for root numbers (Table 3). About the root length, the longest roots formed in *J. curcas* shootlets (4.24 cm) was observed in MS (T25) supplemented with 1.0 mg/l IBA, while, the shortest roots (0.3 cm) were recorded in half MS (T34) supplemented with 3.0 mg/l IBA (Table 3).

In general; the best medium for rooting of *J. curcas* shootlets was half strength MS supplemented with 1.0 mg/l IAA reporting 100% rooting frequency. Also, MS supplemented with IBA gave higher results than that supplemented with IAA. Moreover; half strength MS supplemented with IAA gave a higher value than that supplemented with IBA. In this respect, some workers used MS supplemented with IAA for rooting of *J. curcas* shootlets; Krishna et al. (2010) used MS supplemented with 1.0 mg/l of IAA for subculture of *in vitro* propagated *J. curcas*.

Rajore and Batra (2007) used MS supplemented with 3.0 mg/l IBA for rooting of *in vitro* propagated shootlets of *J. curcas*. Datta et al. (2007) used MS supplemented with 0.2 mg/l IBA for rooting of *in vitro* propagated shootlets of *J. curcas* having 75% root induction. Then they elongated the propagated roots on MS with average length of 8.7 ± 1.35 cm. Thepsamran et al. (2008) cultured *in vitro* produced shootlets of *J. curcas* on MS supplemented with 1.0 mg/l IBA for rooting. Kaewpoo and Te-chato, (2009) reported that, MS supplemented with 0.5 mg/l IBA was the best for rooting of regenerated shootlets of *J. curcas*. Also, Dubey et al. (2010) rooted *in vitro* propagated shootlets of *J. curcas* on MS supplemented with 0.3 mg/l IBA and reported that IBA was more effective than IAA for rooting with rooting frequency of 86.67%.

Some workers used half strength of MS supplemented with IBA only. Chaudhary et al. (1994) sub-cultured the regenerated *J. curcas* plants in half strength of MS supplemented with 0.08 mg/l IBA for rooting, whereas,

Shrivastava and Banerjee (2008) reported that half strength of MS supplemented 3.0 mg/l IBA gave the highest frequency of root induction in the shootlets of *J. curcas*. Moreover, 78% of the propagated shootlets of *J. curcas* were rooted on half MS supplemented with 0.3 mg/l IBA (Li et al. 2008). Recently, Sahoo *et al*. (2012) cultured the regenerated shootlets of *J. curcas* on half MS medium supplemented with 2.0 mg/l IBA for rooting. Also, Verma et al. (2008) successfully rooted *in vitro* propagated shootlets of *J. curcas* using MS and half strength of MS reporting 100% rooting frequency. Moreover, Purkayastha et al. (2010) rooted *in vitro* propagated shootlets of *J. curcas* effectively on half strength MS.

Table 3. Effect of different concentrations of IAA or IBA and medium strength on the rooting of *J. curcas* shootlets after six weeks of culture.

Media codes	MS strength	Growth regulators (mg/l)		Root formation (%)	No. of roots/ shootlet	Root length (cm)
		IAA	IBA			
T1	MS	0	0	-	0.00	-
T25		0	1	90	4.50a	4.24a
T26		0	2	75	3.00b	2.74ba
T27		0	3	90	2.75a	1.26b
T28		1	0	0	0.00	-
T29		2	0	75	0.75cb	2.40ba
T30		3	0	75	0.75cb	1.40b
T31	Half strength MS	0	0	0	0.00c	-
T32		0	1	50	0.50cb	0.63b
T33		0	2	0	0.00	-
T34		0	3	25	0.25dc	0.30cd
T35		1	0	100	3.75a	2.55ba
T36		2	0	75	1.00cb	1.78ba
T37		3	0	90	2.25b	1.80de
$LSD_{0.05}$	(Pr> F 0.001)				0.256	0.889

Within each column, values followed by the same superscript are not significantly different at the p = 0.05 level according to the least significant difference (LSD) test.

Three different soil mixtures have been used to study their effects on acclimation of *J. curcas* plantlets produced from the rooting stage. The maximum percentage of survival of the acclimated plantlets (90) was observed in the soil mixture of sand: peat-moss (1 : 1) Fig. 4A, followed by 85% which was recorded in the soil mixture of sand: peat-moss: perlite (1 : 1 : 1) Fig. 4B. On the other hand, 80% survival was recorded in soil mixture of sand: peat-moss: vermiculate

(1 : 1 : 1) Fig. 4C. In addition, acclimated *J. curcas* plantlet lengths were measured to show the effects of different soil mixtures in the on production of healthy plantlets. The best plantlet length was 15.13 cm observed in the soil mixture of sand : peat-moss (1 : 1) followed by 13.37 cm observed in the soil mixture of sand: peat-moss: perlite (1 : 1 : 1) and 9.7 cm observed in the soil mixture of sand: peat-moss: vermiculate (1 : 1 : 1). Moreover, the highest number of leaves (6.67) was observed in the soil mixture of sand: peat-moss (1:1) followed by 5.0 with that of sand: peat-moss: vermiculate (1 : 1 : 1) and 4.67 with sand: peat-moss: perlite (1 : 1 : 1), respectively.

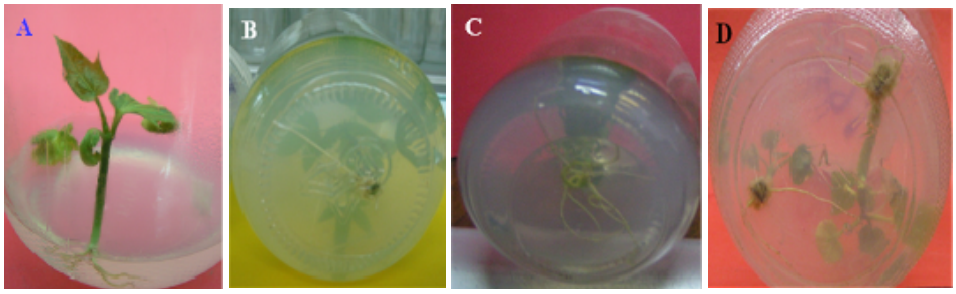

Fig. 3. Rooting of *J. curcas* shootlets on different concentrations of IAA or IBA in MS or half strength of MS; A: Half strength of MS supplemented with 1.0 mg/l IAA, B: MS supplemented with 1.0 mg/l IBA, C: MS supplemented with 3.0 mg/l IBA and D: Half strength of MS supplemented with 3.0 mg/l IAA, after six weeks of culture.

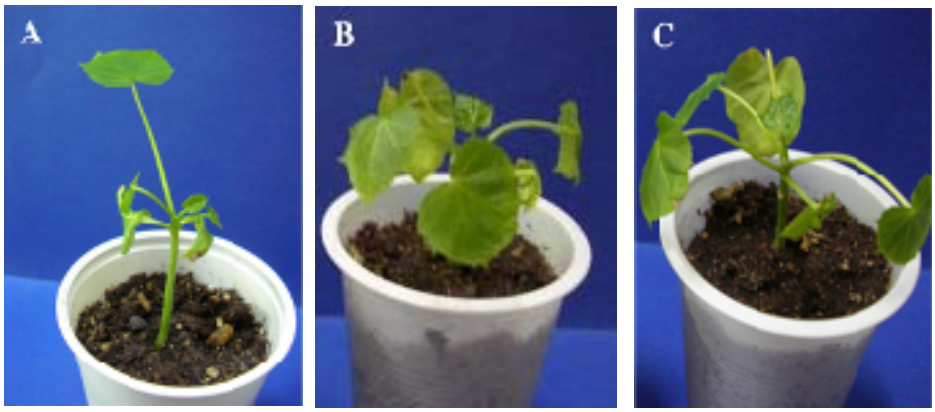

Fig. 4. *In vitro* produced *J. curcas* seedling after one month of their maintenance under greenhouse conditions on; A: sand: peat-moss (1 : 1), B: sand: peat-moss: perlite (1 : 1 : 1) and C: sand : peat-moss: vermiculate (1 : 1 : 1).

Generally, all the treatments nearly gave same results but the soil mixture; sand: peat-moss (1 : 1) was the best mixture for acclimation of *Jatropha*. In this respect, Rajore et al. (2002), acclimated *in vitro* produced *J. curcas* plantlets in the sterilized mixture of soil and vermiculite (3 : 1). They obtained a survival rate of

70 ± 10% while Kalimuthu et al. (2007)**.** Using a mixture of decomposed coir waste, perlite and organic compost in the ratio of (1 : 1 : 1). On the other hand, Purkayastha et al. (2010)**,** produced *in vitro* plantlets with more than 98 % success using soil: compost mixture.

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