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Full Length Research Paper

Efficient callus induction and indirect plant regeneration from various tissues of *Jatropha curcas*

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The *Jatropha curcas* is considered as an important energy plant due to the fact that its seed contains high oil content. Nowadays focus is being placed on *J. curcas* callus induction and plant regeneration. In this study, explants epicotyl, hypocotyl, petiole and cotyledon of 8-day-old seedlings of *J. curcas* were utilized for callus induction on media supplied with 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), naphthyl acetic acid (NAA) or indolebutyric acid (IBA) and 0.1 mg/L kinetin (Kin), and the results demonstrated that the combination of 1 mg/L NAA and 0.1 mg/L Kin was the best medium for callus induction and growth. In addition, induced calli were transferred to regeneration medium containing different combination of auxins and cytokinins, and the data showed that the medium containing 1 mg/L thidiazuron (TDZ) and 1 mg/L Kin combined with 0.1 mg/L IBA was propitious to plant regeneration compared with other combinations.

Key words: Callus induction, indolebutyric acid, *Jatropha curcas,* kinetin, naphthyl acetic acid, plant regeneration, thidiazuron.

INTRODUCTION

Jatropha curcas belonging to the tribe *Jatropheae* in the Euphorbiaceae family is considered as an important energy plant because its seed contains high oil content. *J. curcas* has spread beyond its original distribution due to its hardiness, drought endurance, short gestation period, rapid growth, adoption to wide agro-climatic conditions and multiple uses of different plant parts, which is well adapted to arid and semi-arid climates. It also grows on a large range of soils provided they are well drained and aerated, does not compete arable land with other oleaginous plants or crop plants (Carels, 2009; King et al., 2009; Mukherjee et al., 2011). The seed of *J. curcas* contains 30 to 40% oil with 21% saturated fatty acids and 79% unsaturated fatty acids, and recognition that *J. curcas* oil can yield a high quality biodiesel has led to a surge of interest in *J. curcas* across the globe (Carels, 2009; King et al., 2009). The *J. curcas* oil has a good oxidation stability compared to soybean oil, low viscosity compared to castor oil and a low pour point (the temperature where it starts to become solid) compared to palm oil. The fuel properties of *J. curcas* biodiesel are close to those of fossil diesel and match the American and European standards (Yang et al., 2012). In addition, *J. curcas* is also widely used as folk medicine, fertilizer, manufacturing of soap and candles, illumination, fish poison, inhibitor of watermelon mosaic virus, and nuts collected from a non-toxic Mexican variety are roasted and consumed (Modi et al., 2006; Carels, 2009; Bisen et al., 2010; Mukherjee et al., 2011).

Callus induction and plant regeneration are one of the key tools in plant biotechnology that exploits the totipotent nature of plant cells. Systems of plant regeneration can be categorized as direct and indirect (Mukherjee et al., 2011). Almost all types of explant tissues are now used as regeneration systems through direct (direct generation from explants) and indirect methods (callus-mediated shoot regeneration) (Sujatha and Mukta, 1996; Rajore and Batra, 2005; Varshney and Johnson, 2010; Kumar et

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Abbreviations: BA, Benzylaminopurine; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **GA,** gibberellin; **IAA,** indole-3-acetic acid; **IBA,** indolebutyric acid; **Kin,** kinetin; **MS,** Murashige and Skoog; **NAA,** naphthyl acetic acid; **TDZ,** thidiazuron.

al., 2010a, b). Shoots can be derived either through differentiation of non-meristematic tissues known as adventitious shoot formation or through pre-existing meristematic tissues known as axillary shoot formation. A successful plant regeneration protocol requires appropriate choice of explant, age of the explant, definite media formulations, specific growth regulators, genotype, source of carbohydrate, gelling agent and other physical factors including light regime, temperature, humidity, etc (Sujatha and Mukta, 1996; Sujatha et al., 2005; Deore and Johnson, 2008).

Plant hormones play a crucial role in controlling the way in which plants grow and develop. They regulate the speed of growth of the individual parts and integrate these parts to produce the plants. Both auxins and cytokinins are synergistically required to induce cell division, differentiation and growth in plant tissue cultures (Shrivastava and Banerjee, 2008; Purkayastha et al., 2010; Jha et al., 2007). Many groups have reported that the different combinations of cytokinins such as benzyl-aminopurine (BA), kinetin (Kin) and auxins like naphthyl acetic acid (NAA), indolebutyric acid (IBA) as well as indole-3-acetic acid (IAA) control direct adventitious multiple shoot bud generation from epicotyl, hypocotyl, petiole and cotyledon of *J. curcas* (Cho et al., 2007; Sujatha and Mukta, 1996; Sujatha et al., 2005; Deore and Johnson, 2008).

In direct regeneration system, thidiazuron (TDZ), one of the several substituted ureas that have been investigated recently for their cytokinin-like activity, is known to be more active than zeatin for stimulating the growth, differentiation and organogenesis, especially in direct shoot regeneration, when added to a tissue culture medium at a low concentration (Sujatha et al., 2005; Deore and Johnson, 2008; Kumar and Reddy, 2010; Kumar et al., 2010a, b). In addition to these, Soomro and Memon (2007) used leaf and hypocotyls of *J. curcas* as explants, and found that excellent growth of callus was obtained in medium supplemented with 0.5mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) alone and with 2% (v/v) coconut milk in hypocotyl explants, as well as suspension culture was primarily established. In *J. curcas*, however, there are few reports available on better induction system of callus and regeneration through an intermediary callus phase.

In this study, callus induction and plant regeneration were investigated and the results showed that callus could be excellently induced on appropriate medium from different tissues such as epicotyl, hypocotyl, petiole and cotyledon. The adventitious shoots were efficiently generated from induced callus.

MATERIALS AND METHODS

Seed germination

Seeds of *J. curcas* were collected from Yuanmou, Yunnan Province, China, and stored at room temperature for a year. Seeds were surface-sterilized in 1% $CuSO_4$ for 15 min and rinsed thoroughly with sterile distilled water, and then pre-soaked for imbibition in distilled water for 24 h. The soaked seeds were sowed on six layers of wetted filter papers in trays with covers and germinated in climate chamber at 26°C in the dark for 8 days.

Callus induction

Explants epicotyl, hypocotyl, petiole and cotyledon were isolated from 8-day-old seedlings, and were then sectioned into segments with length 0.5 cm or cm^2 (cotyledon). These segments were disinfected with 75% (v/v) ethyl alcohol for 7 s and then transferred to 0.1% $HgCl_2$ (w/v) solution to sterilize sequentially for 10 min. At the end of sterilization, the segments were rinsed thoroughly with bacteria free water four times, and then transferred to MS (Murashige and Skoog, 1962) medium supplied with the following plant hormones for callus induction, respectively: (1) 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) + 0.1 mg/L kinetin (Kin), (2) 1 mg/L naphthaleneacetic acid (NAA) + 0.1 mg/L Kin, (3) 1 mg/L indolebutyric acid (IBA) + 0.1 mg/L Kin, (4) 0.5 mg/L NAA + 0.1 mg/L Kin, (5) 1.5 mg/L NAA + 0.1 mg/L Kin. Each conical flask contained three segments and cultured in climate chamber at 26°C in the dark for 35 days. The rate of callus induction (%) and fresh weight (g) per callus were counted the 35^{th} day of induction.

Plant regeneration and shoot elongation from callus

The inducted calli by 1 mg/L NAA + 0.1 mg/L Kin from epicotyl were transferred to the following MS medium containing the combination of different plant hormones for plant regeneration, respectively: 0.5, 1 or 2 mg/L thidiazuron (TDZ) + 0.5, 1 or 2 mg/L Kin + 0.05, 0.1 or 0.2 IBA (Table 1). Each conical flask was inoculated three pieces of calli and cultured in climate chamber at 26°C for 45 days, with 150 $\mu mol.m^{-2}.s^{-1}$ and 16 h photoperiod. The number of adventitious shoots per callus and the rate of adventitious shoot induction (%) were counted the 45^{th} day of regeneration (Table 1). Regenerated shoots were transferred to MS medium containing 1 mg/L IAA + 0.5 mg/L gibberellin (GA) to elongate shoots for 30 days.

Rooting and acclimation

The healthy elongated shoots were transferred to 1/2 MS medium containing 0.1 mg/L IBA to root for 20 days, and then the rooting rate was counted. Acclimation of plantlets was carried out according to the methods of Sharma et al. (2011) with a few modifications: Rooted shoots were isolated from medium and washed gently with distilled water several times to remove attached medium, and then transferred to polythene bags containing sterilized soil with perlite, peat and sand (1:2:1) as well as wetted with 0.02% (w/v) carbendazole in greenhouse with 150 $\mu mol.m^{-2}.s^{-1}$ and 16 h photoperiod. The polythene bags were covered with transparent plastic bags to maintain humidity. After 1 week polythene bags were punched, thus decreasing the humidity gradually. After 3 weeks, the established plantlets were transplanted to polybags containing garden soil and farmyard manure, and then transferred to a greenhouse for further growth. The numbers of surviving plants were recorded after 7 weeks.

Statistical analysis

All data were taken from at least three independent experiments. The results were processed statistically using analysis of variance (ANOVA). Figures were drawn by SigmaPlot 11.0, error bars in

Table 1. Effect of the combination of different plant hormones on the formation of shoot buds.

TDZ	Kin	IBA	Number of adventitious shoot per callus	Rate of adventitious shoot induction (%)
0.5	0.5	0.05	2.3 ± 0.15 [d]	15.2 ± 2.0 [d]
1	1	0.1	15.4 ± 0.45 [a]	80.5 ± 3.5 [a]
2	2	0.2	8.2 ± 0.35 [b]	25.3 ± 1.5 [c]
0.5	-	0.05	7.2 ± 0.25 [b]	32.1 ± 2.2 [c]
1	-	0.1	6.4 ± 0.21 [b]	22.4 ± 1.6 [c]
2	-	0.2	6.5 ± 0.25 [b]	21.1 ± 2.0 [c]
0.5	0.5	-	6.3 ± 0.17 [b]	40.4 ± 2.3 [b]
1	1	-	5.1 ± 0.20 [c]	38.5 ± 2.6 [b]
2	2	-	4.7 ± 0.18 [c]	27.2 ± 1.8 [c]
-	0.5	0.05	0	0
-	1	0.1	0	0
-	2	0.2	0	0

Induced calli from epicotyls were transferred to shoot induction medium supplied with the combination of different plant hormones for 45 days. The average values designated different letter express statistically significant differences at the 0.05 level and the same letter are statistically insignificant. TDZ, Thidiazuron; kin, kinetin; IBA, indole-3-butyric acid.

figure represent standard error and each data in figure or table represents the mean ± SE of at least three experiments.

RESULTS

Explants epicotyl, hypocotyl, petiole and cotyledon from 8-day-old seedlings of *J. curcas* were transferred to callus induction medium and cultured in climate chamber at 26°C in the dark for 35 days. The results show that different combinations of plant hormones could induce callus information, and the medium supplied with 1 mg/L NAA and 0.1 mg/L Kin was the most significant among the other mediums; the rate of callus induction reached 100% (Figure 2). Furthermore, appropriate combination of concentration of plant hormone for callus induction, sterilized explants were transferred to the medium with the combination of different concentration of 2,4-D, NAA and IBA, respectively. As shown in Figure 2, different combination of plant hormones could induce the formation of callus and stimulate callus growth, especially in the combination of 1 mg/L NAA and 0.1 mg/L Kin, which was the most significant compared with the other combinations. The fresh weight of this combination was up to 10 g per callus, but another two combinations were 6 and 7 g per callus, respectively. These data implied that different combination of plant hormone could induce the formation of callus in epicotyl, hypocotyl, petiole and cotyledon of *J. curcas* seedlings, and the combination of 1 mg/L NAA and 0.1 mg/L Kin was an appropriate medium for inducing the formation of callus and promoting its growth.

Callus-mediated shoot regeneration is an indirect method for plant regeneration (Datta et al. 2007; Mukherjee et al. 2011). To probe into the formation of adventitious shoot from callus, 35-day-old calli were transferred to plant regeneration medium. The results show that calli from different explants were not different for plant regeneration (data not shown), and calli from epicotyl were used to further plant regeneration experiments due to their rapid growth (Figure 2). At 45 days of plant regeneration, the number of adventitious shoots per callus and the rate of adventitious shoot induction were counted as shown in Table 1. The media containing TDZ could induce the formation of adventitious shoots, but other combinations without TDZ could not, and the number of adventitious shoot per callus and the rate of adventitious shoot induction showed that a trend declined with the increased concentration of TDZ. In addition, the medium supplied with 1 mg/L TDZ, 1 mg/L Kin and 0.1 mg/L IBA not only increased the number of adventitious shoots per callus, but also improved the rate of adventtious shoot induction among the eleven regeneration medium. These results suggested that plant regeneration media containing TDZ in combination with other plant hormones could stimulate the formation of adventitious shoots and the combination of 1 mg/L TDZ, 1 mg/L Kin and 0.1 mg/L IBA was a suitable medium for adventitious shoots from callus.

After shoot elongated, the healthy regenerated shoots were rooted on 1/2 MS medium supplied with 0.1 mg/L IBA and rooting rate reached above 90%. Regenerated plants with well developed shoots and roots were successfully acclimated in soil, and then transferred to greenhouse for further growth. Moreover, there were no visible detectable variations during the process of growth (Figure 1).

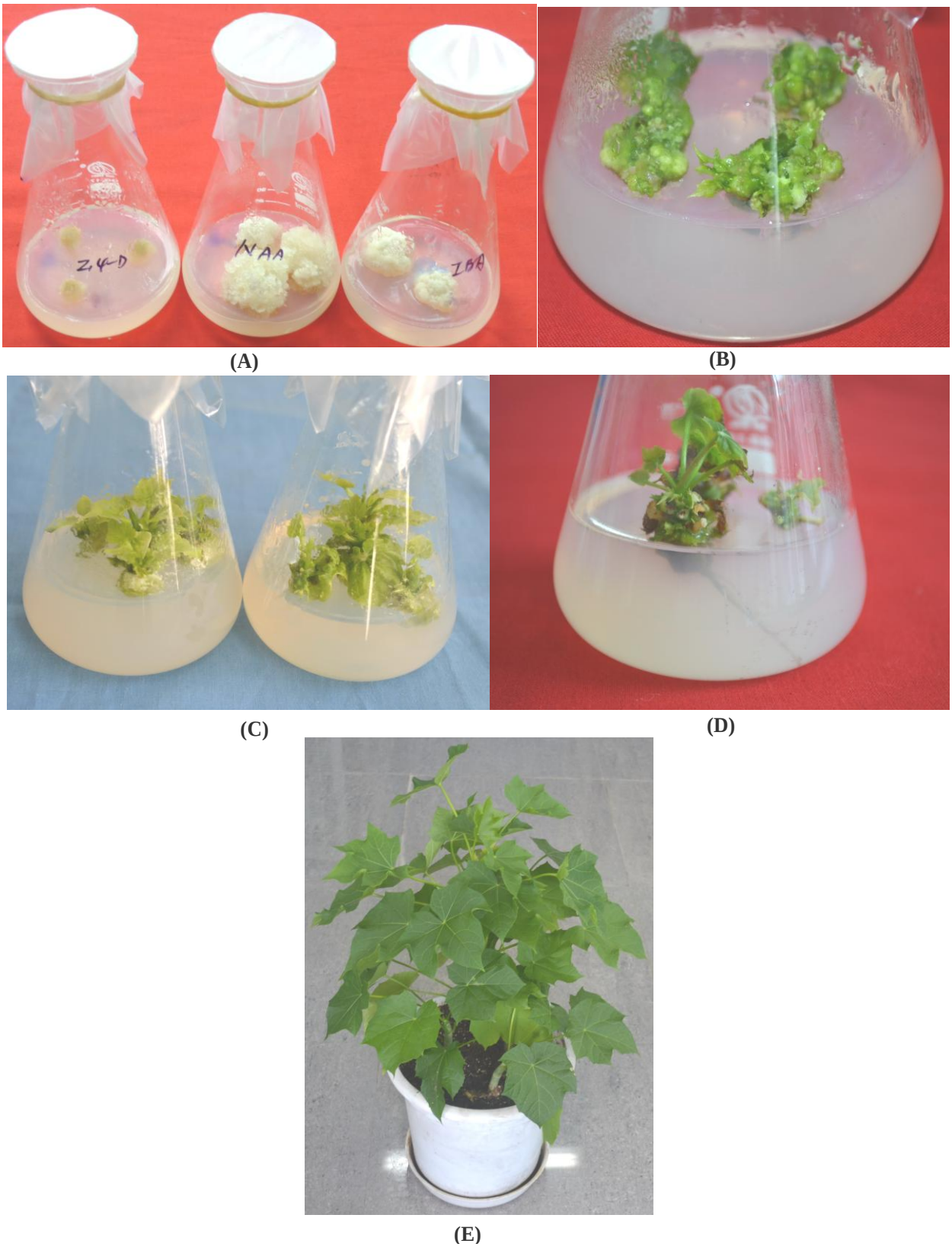

Figure 1. Callus induction and plant regeneration. (**A)** Callus induction of epicotyl in MS supplied with 1 mg/L 2,4-D,NAA or IBA and 0.1 mg/L Kin for 35 days. (**B)** Formation of shoot buds from callus in MS supplied with 1 mg/L TDZ, 1 mg/L BA and 0.1 mg/L Kin for 45 days. (**C)** Propagation and elongation of shoots. (**D)** Rooting of shoots. (E) Rooted plants transplanted to pot for 4 months.

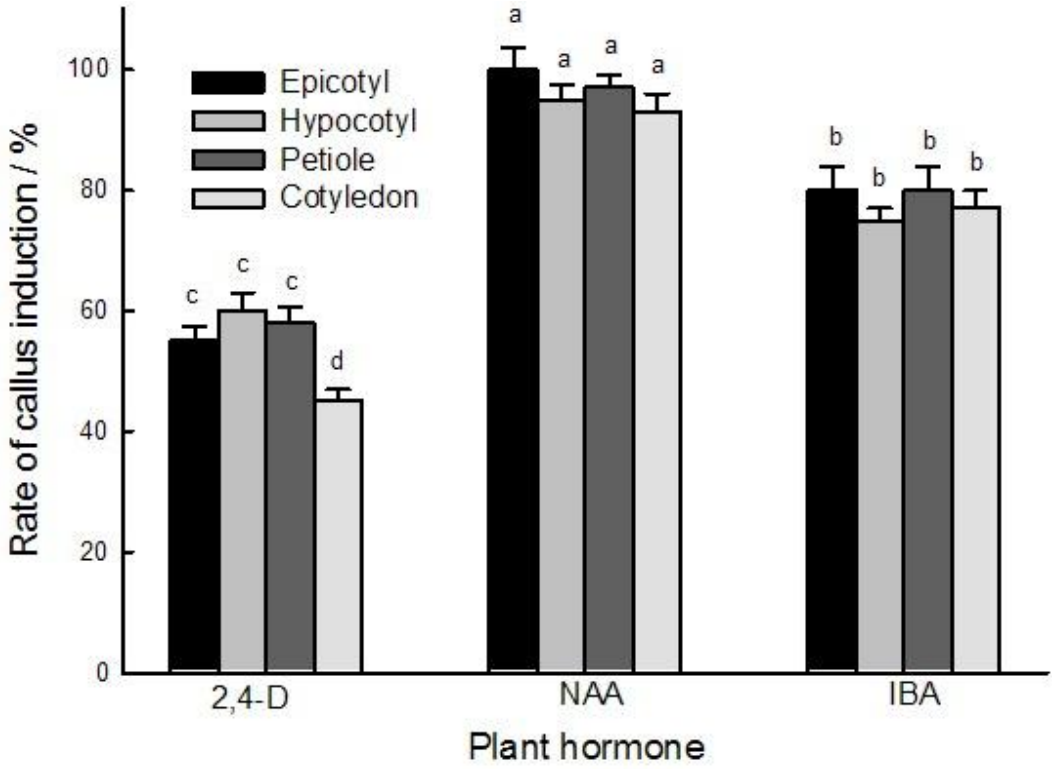

Figure 2. Effect of the combination of different plant hormones on induction and growth of callus. Explants epicotyl, hypocotyl, petiole and cotyledon were transferred to callus induction medium supplied with 1 mg/L 2,4-D,NAA or IBA and 0.1 mg/L Kin for 35 days. Error bars represent standard error and each data in figure represents the mean ± SE of three experiments. The bars designated with different letter express statistically significant differences at the 0.05 level and the same letter are statistically insignificant.

DISCUSSION

Interaction of auxins and cytokinins plays vital role in cell division, growth, development, differentiation and the formation of plant organs (Shrivastava and Banerjee, 2008; Purkayastha et al., 2010; Jha et al., 2007). The present results showed that different combination of plant hormone could induce the formation of callus in epicotyl, hypocotyl, petiole and cotyledon of *J. curcas* seedlings, and the combination of 1 mg/L NAA and 0.1 mg/L Kin was an appropriate medium for inducing the formation of callus and promoting its growth (Figures 2 and 3). At the same time, plant regeneration media supplied with TDZ in combination with other plant hormones could stimulate the formation of adventitious shoots and the combination of 1 mg/L TDZ, 1 mg/L Kin and 0.1 mg/L IBA was a suitable medium for adventitious shoots from callus.

A number of studies have showed that auxin, such as 2,4-D, IBA and NAA play an important role in callus induction (Soomro and Memon, 2007; Lu et al., 2003; Qin et al., 2006; Wei et al., 2004). In *J. curcas*, Soomro and Memon (2007) have found that excellent growth of callus was obtained in medium supplemented with 0.5 mg/L 2, 4-D alone and with 2% (v/v) coconut milk in hypocotyl explants; the callus produced from hypocotyl explants grew faster during 7 to 30 days of culture then stabilized at a low growth rate, and calli cultured on this medium showed an 8-fold increase in fresh weight by the fourth week of incubation. In a work carried out by Lu et al. (2003), it was found that MS medium supplied with 0.5 mg/L BA and 1 mg/L IBA is the best medium for callus induction from hypocotyl and petiole of *J. curcas*. In addition, Wei et al. (2004) used plumules, cotyledons, hypocotyls, leaf blades, petioles and stalks of *J. curcas* as explants, and found that the MS medium with 5 mg/L BA and 1 mg/L NAA was the best for callus induction. In this experiment, the combination of 1 mg/L NAA and 0.1 mg/L Kin was an appropriate medium for inducing the formation of callus and promoting its rapid growth in epicotyl, hypocotyl, petiole and cotyledon of *J. curcas* seedlings (Figures 2 and 3). These data implied that growth hormone, especially NAA, plays a key role in excellent induction and growth of callus in *J. curcas*, which may be involved in that NAA is easy to enter into the cell (Oono et al., 2003).

TDZ is one of the several substituted ureas that have been investigated recently for their cytokinin-like activity, which is known to be more active than zeatin for

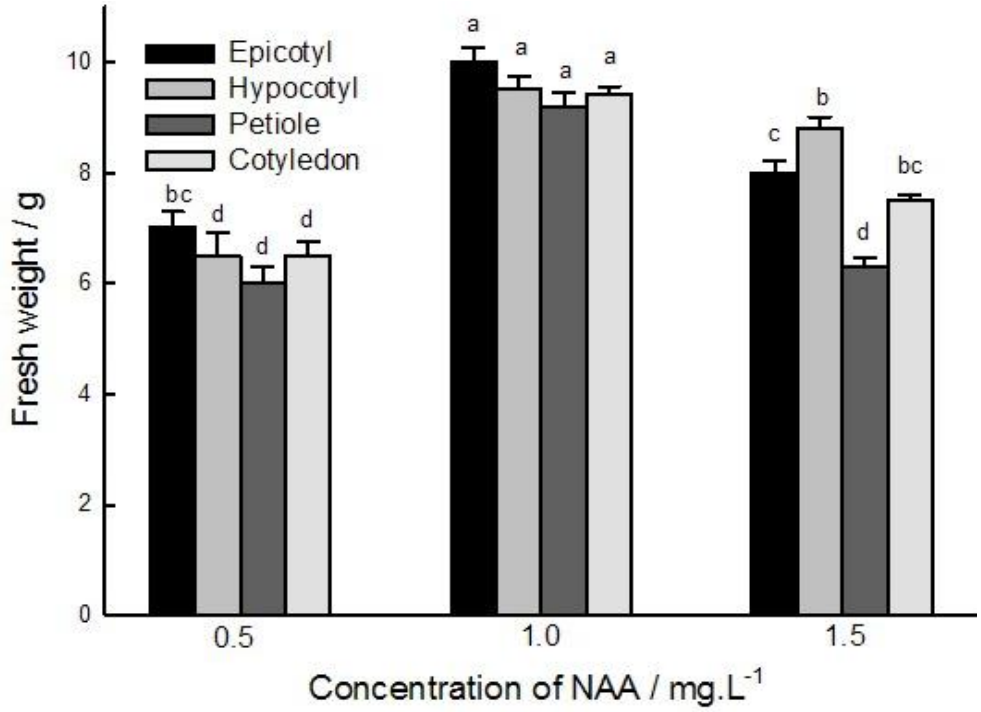

Figure 3. Effect of the combination of different concentration of auxins on induction and growth of callus. Explants epicotyl, hypocotyl, petiole and cotyledon were transferred to callus induction medium supplied with 0.5, 1 or 1.5 mg/L NAA and 0.1 mg/L Kin for 35 days. Error bars represent standard error and each data in figure represents the mean ± SE of three experiments. The bars designated different letter express statistically significant differences at the 0.05 level and the same letter are statistically insignificant.

stimulating the growth when added to a tissue culture medium at a low concentration (Kim et al., 2001; Sujatha et al., 2005; Deore and Johnson, 2008; Kumar and Reddy, 2010; Kumar et al., 2010a, b). Deore and Johnson (2008) found that adventitious shoot buds were induced from very young leaf explants of *in vitro* germinated seedlings as well as mature field-grown plants cultured on MS medium supplemented with 2.27 µM TDZ, 2.22 µM BA and 0.49 µM IBA, and the presence of TDZ in the induction medium has greater influence on the induction of adventitious shoot buds. In addition, Sharma et al. (2011) also reported that the best results irrespective of genotype were obtained on the medium containing 0.5 mg/L TDZ and *in vitro* hypocotyl explants were observed to have higher regeneration efficiency as compared to *ex vitro* explant in both toxic and non-toxic genotypes.

In addition to these, the study of Kumar et al. (2011) indicated that maximum regeneration efficiency (81.07%) and the number of shoot buds per explants was observed on 9.08 µM TDZ containing MS medium from *in vitro* cotyledonary leaf explants. Our present results also showed that plant regeneration media supplied with TDZ in combination with Kin or IBA could stimulate the formation of adventitious shoots, and the combination of 1 mg/L TDZ, 1 mg/L Kin and 0.1 mg/L IBA was a best medium for adventitious shoot formation (Table 1). These results suggest that TDZ plays a very important role in the formation of adventitious shoot buds of *J. curcas*, and these effects may be involved in stimulating *de novo* synthesis of auxins by increasing the levels of IAA and its precursor, tryptophan, as well as increase in contents of endogenous cytokinin and ethylene (Murthy et al., 1995; Murthy and Saxena, 1998). The other possibilities include the modification in cell membranes, energy levels, nutrient uptake, or nutrient assimilation (Murthy et al., 1995,Murthy and Saxena; 1998).

In conclusion, it is clearly shown that the system of excellent induction and growth of callus in epicotyl, hypocotyl, petiole and cotyledon of *J. curcas* seedlings is the medium supplied with 1 mg/L NAA and 0.1 mg/L Kin, and the combination of 1 mg/L TDZ, 1 mg/L Kin and 0.1 mg/L IBA is the best plant regeneration system. Regenerated shoots could induced root formation on 1/2 MS medium with 0.1 mg/L IBA, and survival percentage reached above 90% through acclimating in polythene bags.

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