Research Article

Regeneration of Adventitious Shoots from Callus and Leaf Explants in *Jatropha curcas* L. 'Phetchaburi'

Pantipa Na Chiangmai*, Yupa Pootaeng-on, Pimjai Meetum, Kanokwan Kamkajon, Waraporn Yuiam, Narumon Rungphan and Panwadee Ninkaew

Faculty of Animal Science and Agricultural Technology, Silpakorn University, Phetchaburi IT Campus, Cha-Am, Phetchaburi, Thailand
**Corresponding author. Email address: mchiangmai@gmail.com*

Received December 17, 2013; Accepted July 21, 2014

Abstract

The effect of cytokinins and auxins was evaluated in inducing a physic nut (*Jatropha curcas* L.) callus while the effect of the combinations of cytokinin and either indole-3-butyric acid (IBA) or some growth additives was determined in regenerating adventitious shoot from a leaf segment. The results showed MS medium containing only 0.1 or 1.0 mg/l thidiazuron (TDZ) was the most effective medium for inducing morphogenic callus. MS medium supplemented with 2.0 mg/l 6-benzylaminopurine (BA) and 0.5 mg/l indole-3-acetic acid (IAA) gave the highest shoot regeneration percentage and shoot height from the callus. On the other hand, the MS medium supplemented with 0.5 mg/l BA and 1.0 mg/l IBA was the most effective medium for adventitious shoot regeneration and shoot height from the leaf segments.

***Key Words*:** Physic nut; Plant growth regulators; Shoot regeneration; Tissue culture explants

Introduction

Jatropha curcas L. is one of oil seed plant species, commonly known as the physic nut. The plant has high oil content in seed and its resistance to drought and infertile soil (Heller, 1996; Winkler et al., 1997).

Although physic nut is easy to propagate by stem cutting, mass propagation under aseptic technique is needed for genetic improvement and disease-free plantlet multiplication. Various kinds of material such as plantlets, explants, callus and protoplast were used for genetic improvement and genetic study (Bhansali, 1990; Sujatha and Mukta, 1996; Wei et al., 2004; Jha et al., 2007; Divakara et al., 2009; Maharana et al., 2012).

Type of explants and concentration of plant growth regulators in tissue culture medium for shoot regeneration were studied in physic nut from different genetic backgrounds (Sujatha and Mukta, 1996; Sardana et al., 2000; Wei et al., 2004; Rajore and Batra, 2005). Callus and cotyledon were utilized as an explant for study in genetic variation and transformation as well as toxic compound reduction in physic nut (Li et al., 2007; Pan et al., 2010; Wirasutisna et al., 2011; Jose et al., 2012; Subroto et al., 2014). The cotyledonary leaf explant was also reported recently as the mediate transformation in physic nut (Mazumdar et al., 2010).

The combinations among plant growth

regulators or between plant growth regulator and amino acid were the most effective medium for inducing shoot from leaf explants in MS (Murashige and Skoog, 1962) supplemented with 0.45 µM (0.1 mg/l) thidiazuron (TDZ) and 300 mg/l proline or 500 mg/l casein hydrolysate (Burikam and Kawita, 2007). Other supplements in the MS mediums induced high percentage of shoot regeneration from various explants, such as epicotyl, hypocotyl and petiole explants. These supplements included 2.22 µM (0.5 mg/l) 6-benzylaminopurine (BA) and 0.49 µM (0.1 mg/l) indole-3-butyric acid (IBA) (Wei et al., 2004) or 4.90 µM (≈1 mg/l) IBA (Sujatha and Mukta, 1996). However, BA (13.3 µM) and IBA (2.46 µM) in MS mediums were effective in inducing callus and shoot bud formation from leaf explant (Khurana-Kaul et al., 2010).

Apart from type of explants and concentration of plant growth regulators, genetic of the phytic nut plant may play a role in the efficiency of shoot regeneration. This study aimed to evaluate the effect of plant growth regulators in regenerating shoot in *J. curcas* (var. Phetchaburi) which has been utilized for oil extraction in Thailand.

Materials and Methods

Plant materials

In Experiment 1, the effect of types and concentrations of plant growth regulators in culture media on shoot induction from 2-week-old callus second subculture was evaluated.

In vitro seedlings of 'Phetchaburi' physic nut by culturing seeds on MS medium for 2 weeks. The hypocotyls of seedlings were excised (0.5 cm in length) for callus induction on MS medium supplemented with 1.0 mg/l BA and 0.5 mg/l IBA for 3 weeks. Hypocotyl-derived callus was further sub-cultured and maintained for 3 weeks for the following experiments.

In Experiment 2, the effect of combinations of either between IBA or some growth additives (proline and casein hydrolysate) and cytokinins in MS medium on shoot induction from leaf explants was evaluated. The leaf explants from aseptic seedling were excised to attain 0.5 cm x 0.5 cm pieces.

Experimental treatments

In Experiment 1.1, callus was cultured on various shoot regeneration media. The effects of cytokinin types including BA, kinetin (KN) and TDZ, and auxin (IBA) on shoot induction from callus were studies. Ten combination treatments of cytokinins and auxin were conducted including 1) MS (control treatment); 2) MS + 0.1 mg/l TDZ; 3) MS + 1.0 mg/l TDZ; 4) MS + 2.0 mg/l BA; 5) MS + 2.0 mg/l KN; 6) MS + 1.5 mg/l IBA; 7) MS + 0.1 mg/l TDZ + 1.5 mg/l IBA; 8) MS + 1.0 mg/l TDZ + 1.5 mg/l IBA; 9) MS + 2.0 mg/l BA + 1.5 mg/l IBA; and 10) MS + 2.0 mg/l KN + 1.5 mg/l IBA.

In Experiment 1.2, callus was cultured on four shoot regeneration media including 0.5 mg/l BA + 1.0 mg/l IBA; 0.5 mg/l BA + 0.01 mg/l IBA; 2.0 mg/l BA + 0.5 mg/l IAA (indole-3-acetic acid); and 3.0 mg/l BA + 3.0 mg/l IAA in MS medium. The concentrations of plant growth regulators which had been used in this study were employed based upon their capacity to induce the regeneration of embryogenic callus and shoot from explants of physic nut (Sujatha and Mukta, 1996; Sardana et al., 2000; Thepsamran et al., 2008; Kapil and Sharma, 2014).

In both Experiment 1.1 and 1.2, one piece of callus was cultured on each shoot regeneration medium in a bottle (size 125 ml or 4 oz), 15 and 10 bottles as one replication, respectively, and 4 and 5 replications were conducted in these experiments, respectively.

In Experiment 2, leaf explant was cultured on four shoot regeneration media. MS medium was supplemented with 0.1 mg/l TDZ + 300 mg/l proline; 0.1 mg/l TDZ + 500 mg/l casein hydrolysate; 0.5 mg/l BA + 0.1 mg/l IBA; and 0.5 mg/l BA + 1.0

mg/l IBA. The concentrations of plant growth regulators which had been used in this study were employed based upon their capacity to induce the regeneration of shoot from explants such as leaf and axillary bud of physic nut (Sujatha and Mukta, 1996; Wei et al., 2004; Burikam and Kawita, 2007; Thepsamran et al., 2008).

Ten pieces of young leaf explant (0.5 cm x 0.5 cm in size) were excised from a seedling and placed in a petri-dish for shoot induction and 5 petri-dishes as one replication. Four replications were conducted in this experiment.

Characteristic determination

In Experiment 1.1, some characteristics of callus were determined 45 days after cultured on shoot induction medium. The characteristics included percentage of yellow/green compact or friable callus after transferred to shoot regeneration medium, percentage of differentiated callus which was observed from the morphological changes either on proliferation or shoot regeneration and percentage of differentiated callus which was only observed on shoot regeneration.

In Experiment 1.2, some characteristics were determined 30 days after cultured callus on shoot induction medium. These characteristics included the percentage of shoot regenerated callus and shoot height.

In Experiment 2, some characteristics, such as percentage of green leaf segment explants after transferring to shoot induction medium, percentage of shoot regeneration per green leaf explant and shoot height, were determined 28 days after cultured callus.

Experimental design and data analysis

For experiment 1.1, the 5 x 2 factorial in Completely Randomized Design (CRD) was arranged for shoot regeneration from callus with 4 replications. Factor A was types and concentrations of cytokinins (none, 0.1 mg/l TDZ, 1.0 mg/l TDZ, 2.0 mg/l BA, and 2.0 mg/l KN). Factor B was the present or absent of 1.5 mg/l IBA. These factors (both A and B) were applied in MS medium for shoot regeneration. For experiment 1.2 and 2, experiments were set up in CRD with 5 and 4 replications, respectively.

Data of all characteristics were subjected to the analysis of variance (ANOVA), in which the F-test was used to test the significant differences. The Duncan's New Multiple Range Test (DMRT) was used for treatments comparing after significant difference of variance by ANOVA was observed.

All experiments were conducted in a constant temperature at 26° C and 16 hours photoperiod illuminated with florescent tube of 25,000 lux in plant tissue culture laboratory, Faculty of Animal Science and Agricultural Technology, Silpakorn University, Phetchaburi Information Technology (IT) Campus, Phetchaburi province, Thailand.

Results

Effect of plant growth regulators on callus

Callus was placed on MS medium supplemented with different concentrations of cytokinin and auxin exhibited the morphological changes involved to shoot regeneration. The callus was watery, brown, necrotic, and compact yellow/green callus (not found the friable callus) which displayed after transferring to shoot induction medium (data not shown).

In Experiment 1.1, highly significant difference ($P<0.01$) was observed on yellow/green compact callus after cultured on shoot induction medium containing cytokinins (TDZ, BA and KN), IBA and the interaction between them (Table 1). The results showed that highest yellow/green compact callus percentage was found in the MS mediums supplemented with either 0.1 mg/l or 1.0 mg/l TDZ treatment. IBA (at 1.5 mg/l) in MS mediums had higher percentage of yellow/green compact callus

Table 1 Percentage of yellow/green compact callus (mean ± SD) induced from 'Phetchaburi' physic nut callus after 45 days on different shoot induction MS media

PGR	none	0.1 mg/l TDZ	1.0 mg/l TDZ	2.0 mg/l BA	2.0 mg/l KN	Mean ± SD‡
0 mg/l IBA	65.0 ± 28.0‡ b	91.7 ± 17.6 a	98.3 ± 6.4 a	68.3 ± 17.6 b	11.7 ± 24.8 c	67.0 ± 18.9 y
1.5 mg/l IBA	98.3 ± 6.4 a	96.7 ± 12.9 a	95.0 ± 10.4 a	93.3 ± 19.8 a	95.5 ± 10.1 a	95.8 ± 11.9 x
Mean ± SD‡	81.7 ± 17.2 n	94.2 ± 15.2 m	96.7 ± 8.4 m	80.8 ± 18.7 n	47.1 ± 17.4 o	

‡ significant difference at the 0.01 level of probability.

Table 2 Percentage of differentiated callus either proliferate or had shoot regeneration (mean ± SD) induced from 'Phetchaburi' physic nut callus after 45 days on different shoot induction MS media

PGR	none	0.1 mg/l TDZ	1.0 mg/l TDZ	2.0 mg/l BA	2.0 mg/l KN	Mean±SD‡
0 mg/l IBA	46.7±29.7‡ d	85.0±22.8 abc	80.0±23.5 bc	51.7±22.1 d	5.0±14.0 e	53.7±22.4 y
1.5 mg/l IBA	96.7±8.8 a	96.7±12.9 a	95.0±10.4 ab	93.3±20.0 ab	72.7±26.1 c	91.9±15.6 x
Mean±SD‡	77.7±19.2 n	90.8±17.8 m	87.5±16.9 m	72.5±21.0 n	33.6±20.1 o	

‡ significant difference at the 0.01 level of probability.

(95.8%) than un-treated control (67%) (Table 1).

Although callus formation was significantly different among the interaction between concentrations/ types of cytokinin and the present/ absent of IBA (auxin), high percentage of yellow/ green compact callus was found in the MS mediums containing TDZ (at both 0.1 and 1.0 mg/l concentrations) (Table 1). The lowest percentage of yellow/green compact callus (11.7%) was found in MS mediums supplemented with 2.0 mg/l KN (Table 1).

Differentiated callus was observed from the change on morphology either to proliferate or had regenerated shoot at 45 days after replaced callus on shoot induction medium. The differentiated callus was significantly affected by supplementing MS medium with cytokinins (TDZ, BA and KN) and auxin (IBA), and the interaction between them (Table 2).

The results showed the high percentage of differentiated callus on MS mediums supplemented either 0.1 mg/l or 1.0 mg/l TDZ. The present of 1.5 mg/l IBA in MS mediums increased differentiated callus (91.9%) compared with un-treated control (53.7%) (Table 2).

However, the different increasing of percentage of differentiated callus was observed on 1.5 mg/l IBA supplemented MS medium combined with different cytokinin types (TDZ, BA or KN) (Table 2). MS medium supplemented both of 1.5 mg/l IBA and 0.1 mg/l TDZ had highest value of differentiated callus (96.7%), while MS medium supplemented with 2.0 mg/l KN had the lowest value (5%) (Table 2).

The combination of cytokinin and auxin increased percentage of differentiated callus. MS medium supplemented only with 0.1 mg/l TDZ had differentiated callus (85%) which was not statistically significantly different to the medium supplemented with both 0.1 mg/l TDZ and 1.5 mg/l IBA (96.7%) (Table 2).

Shoot regeneration from differentiated callus was determined 45 days after transferred callus to shoot induction medium. The shoot regeneration percentage was less than 1% in all combinations (Table 3).

The effect of the combinations of cytokinins

Table 3 Percentage of differentiated callus proliferate which had shoot regeneration (mean ± SD) induced from 'Phetchaburi' physic nut callus after 45 days on different shoot induction MS media

PGR	none	0.1 mg/l TDZ	1.0 mg/l TDZ	2.0 mg/l BA	2.0 mg/l KN	Mean±SD[ǂ]
0 mg/l IBA	0.0±0.0[ǂ b]	0.3±0.6 [a]	0.3±0.6 [a]	0.0±0.0 [b]	0.0±0.0 [b]	0.1±0.2 [a]
1.5 mg/l IBA	0.0±0.0 [b]	0.0±0.0 [b]	0.0±0.0 [b]	0.0±0.0 [b]	0.0±0.0 [b]	0.0±0.0 [b]
Mean±SD[λ]	0.0±0.0	0.2±0.3	0.2±0.3	0.0±0.0	0.0±0.0	

[λ]Ns, not significant difference at the 0.05 level of probability

[ǂ] significant difference at the 0.01 level of probability

Table 4 Percentage of shoot regenerated callus and shoot height (mean ± SD) characteristics induced from 'Phetchaburi' physic nut callus after 30 days on different shoot induction MS media

Shoot induction medium	Percent of shoot regenerated callus (%)	Shoot height (cm)
MS + 0.5 mg/l BA + 1.0 mg/l IBA	32.23 [c]	1.02 ± 0.85 [b]
MS + 0.5 mg/l BA + 0.01 mg/l IBA	49.05 [b]	1.98 ± 0.36 [a]
MS + 2.0 mg/l BA + 0.5 mg/l IAA	81.30 [a]	2.15 ± 0.32 [a]
MS + 3.0 mg/l BA + 3.0 mg/l IAA	00.00 [d]	Nd
F-test	**	**

Nd, not include for ANOVA

** significant difference at the 0.01 level of probability

(TDZ, BA and KN) and auxin (1.5 mg/l IBA) on the regeneration of shoot was highly significant. The MS mediums contained only TDZ (at both 0.1 and 1.0 mg/l) increased shoot regeneration (Table3).

In Experiment 1.2, the percentage of shoot regenerated callus and shoot height at 30 days after transferred to shoot induction medium were shown in Table 4.

Percentage of shoot regenerated callus was highly affected by MS mediums supplemented with various concentrations and types of cytokinin/auxin (Table 4). The highest percentage of shoot regenerated callus was observed in MS medium supplemented with 2.0 mg/l BA and 0.5 mg/l IAA at 81.30%, followed by MS mediums supplemented with 0.5 mg/l BA and 0.01 mg/l IBA (49.05%), and 0.5 mg/l BA and 1.0 mg/l IBA (32.23%). However, MS supplemented with 3.0 mg/l BA and 3.0 mg/l IAA did not induce shoot regeneration from callus culture (Table 4).

Shoot height was determined and presented in Table 4. MS mediums supplemented with the combinations of 2.0 mg/l BA and 0.5 mg/l IAA (2.15 cm), and the combination of 0.5 mg/l BA and 0.01 mg/l IBA (1.98 cm) highly affected shoot height (Table 4).

Effect of growth regulators on leaf segment explants on shoot regeneration

In Experiment 2, percentage of green leaf segment explants, percentage of shoot initiation and shoot height, were determined (Table 5). These were evaluated based on green leaf explant numbers. The percentage of green leaf segment explant was not significantly different among four shoot induction mediums (Table 5).

Percentage of regenerated shoot from green leaf segment explant was affected by different supplements in the MS mediums (Table 5). The highest value of percentage of regenerated shoot was found in the mediums containing the

Table 5 Percentage of green leaf explants of physic nut (mean ± SD) was evaluated 28 days after transferred by upside down on different shoot induction mediums

Shoot induction medium	Percent of green leaf explants	Percent of regenerated shoots per green leaf explants	Shoot height (cm)
MS + 0.1 mg/l TDZ + 300 mg/l proline	65.0 ± 28.9	7.7 ± 15.4 [a]	0.4 ±0.1 [b]
MS + 0.1 mg/l TDZ + 500 mg/l casein hydrolysate	35.0 ± 23.8	0.0 ± 0.0 [b]	Nd
MS + 0.5 mg/l BA + 0.1 mg/l IBA	55.0 ± 26.3	4.6 ± 9.1 [b]	0.2 ± 0.2 [c]
MS + 0.5 mg/l BA + 1.0 mg/l IBA	80.0 ± 40.0	6.3 ± 12.5 [a]	0.6 ± 0.3 [a]
F-test	Ns	*	*

Nd, not include for ANOVA
Ns, not significant difference at the 0.05 level of probability
* significant difference at the 0.05 level of probability

combination of 0.1 mg/l TDZ and 300 mg/l proline (7.7%), and the combination of 0.5 mg/l BA and 1.0 mg/l IBA (6.3%) (Table 5), followed by the MS mediums supplemented with the combination of 0.5 mg/l BA and 0.1 mg/l IBA (4.6%), and the combination of 0.1 mg/l TDZ and 500 mg/l casein hydrolysate (0%) (Table 5).

Shoot height was highly affected by shoot induction mediums. The highest value of plant height was observed in MS medium supplemented with 0.5 mg/l BA and 1.0 mg/l IBA at 0.6 cm, followed by MS mediums supplemented with the combination of 0.1 mg/l TDZ and 300 mg/l proline (0.4 cm), and the combination of 0.5 mg/l BA and 0.1 mg/l IBA (0.2 cm) (Table 5).

Discussion

Callus induction and shoot regeneration were affected by factors such as types of explants, culture mediums, plant growth regulators, condition during culturing and development stage of explants (Yeoman and Yeoman, 1996; Ahmed et al., 2011; Zalewska et al., 2011). Both friable and compact callus were successfully induced by various researches (Hutchinson et al., 1997; Lin et al., 2000; Kaewpoo and Te-chato, 2009).

The result showed the effects of concentration and combination of plant growth regulators (cytokinin and auxin) on shoot regeneration by transforming yellow/green callus to plantlet regeneration was similar to the result reported in physic nut (Biradar et al., 2012; Jose et al., 2012). Media containing IBA (at 1.5 mg/l) showed higher yellow/green compact callus percentage than that of the medium without IBA (Table 1). It was reported that white to light green callus of physic nut was observed in medium contained IBA at a low concentration (Jose et al., 2012).

The higher percentage of yellow/green compact callus was found in treatments contained the combination of cytokinin (BA and KN) and IBA. On the contrary, the media containing a combination of IBA (0.1-0.4 mg/l) and BA (0.5-2.5 mg/l) induced higher callus formation in physic nut (Sujatha and Mukta, 1996). The media supplemented only with BA did not affect callus formation in physic nut (Jose et al., 2012).

The results in this study showed that high percentage of yellow/green callus was obtained in MS medium supplemented with TDZ (at 0.1 and 1.0 mg/l) (Table 1). This result was similar to the research reported by Sujatha and Mukta (1996)

which showed that white and green compact callus in physic nut could be obtained in TDZ-contained medium (at 1-3 mg/l). TDZ was found to have an effect to plant similar to auxin and cytokinin (Murthy et al., 1998; Guo et al., 2011), although it had the chemical structure different from these two plant hormones (Murthy et al., 1998; Guo et al., 2011). TDZ may involve in modulating endogeneous plant hormones, resulting to the modification of plant cell membranes and uptake and assimilation of plant nutrient (Murthy et al., 1998). For these reasons, TDZ was used to study in various plant species and explant types (Murhy et al., 1998; Ahmed and Anis, 2012; Sharma et al., 2012).

Callus was transformed to shoot in the induction medium (Table 2), indicating the biochemical and physiological occurrence within cells of this tissue. The high percentage of differentiated callus was obtained in MS medium supplemented with various types of cytokinin (TDZ, BA and KN). These cytokinin derivatives were reported to promote shoot proliferation (Sujatha and Reddy, 1998). However, TDZ (at 0.1 and 1.0 mg/l) was more effective than KN and BA in regenerating shoot of physic nut in this study and other plant species (Sujatha and Reddy, 1998; Gallo-Meagher et al., 2000; Verma et al., 2011; Karataş et al., 2013). The plantlet regeneration was very low in this experiment (Table 3). However, MS medium supplemented with TDZ was the only medium which showed the positive result for adventitious shoot regeneration. The high concentration of TDZ supplemented in the medium and the duration of culturing plant tissue in this medium may affect induction and elongation of the shoot (Huetteman and Preece, 1993; Lu, 1993; Murthy et al., 1998; Karataş et al., 2013). However, low percentage of regeneration was observed in this study when the tissues were cultured in the MS medium supplemented with low concentration of TDZ (0.1 and 1.0 mg/l). Other factors such as genotype and types of an explant may affect plant regeneration and shoot elongation in physic nut (da Camara Machado et al., 1997; Kumar and Reddy, 2010).

The result in Experiment 1.2 showed that MS medium supplemented with different kinds and concentrations of plant growth regulator may affect callus with respect to the percentage of shoot induction and shoot height (Table 4). This finding was in agreement of the previous works as reported by Sujatha and Mukta (1996) and Sujatha and Reddy (1998) in physic nut and castor.

An increase in shoot regeneration from callus was obtained in MS medium supplemented with decreased IBA (at 0.01 mg/l and 1.0 mg/l) and BA (0.5 mg/l) concentrations (Table 4). The combination of BA (0.5 mg/l) and IBA (0.25 mg/l) was reported to induce shoot of physic nut from various explant types (axillary bud, shoot tip and stem) and callus (Sujatha and Dhingra, 1993; Sujatha and Mukta, 1996; Kaewpoo and Te-chato, 2009).

Both low and high concentrations of auxin and cytokinin affected shoot regeneration and shoot elongation, respectively (Kaewpoo and Te-chato, 2009). However, few shoot would be obtained from the callus derived from hypocotyl of physic nut (Kaewpoo and Te-chato, 2009). In this study, lower shoot regeneration was obtained on hypocotyl-derived callus in the MS medium supplementing with the mixture of BA and IBA (Table 4).

IAA affected shoot development both from shoot bud, leaf cotyledon explant and callus when MS medium supplemented with BA (Singh et al., 2010; Nunes et al., 2013; Biradar et al., 2012). The concentration of IAA at 1.49 mg/l (or 8.5 μM) and BA at 0.51 mg/l (or 2.25 μM) in the MS medium was reported effective for elongating shoot when petiole explant of physic nut was used in the experiment (Kumar and Reddy, 2010). For shoot regeneration, a combination of IAA (at 0.5 mg/l)

and BA (at 2.0 mg/l) increased plant height in Experiment 1.2 (Table 4). However, higher concentration of IAA (at 3.0 mg/l) and BA (at 3.0 mg/l) did not induce shoot (Table 4). High concentration of kinetins and auxins was reported to have a negative effect on the shoot regeneration (Preece and Imel, 1991; Palai et al., 1997; Raghu et al., 2006; Premkumar et al., 2011).

In Experiment 2, after leaf explants were transferred to shoot induction medium, some tissues were pale, brown, necrotic and green leaf explants (data not shown). Three (out of in four leaf) explants did not regenerate to shoot.

Only the green leaf segment explants were found to transform to shoot. The success of shoot formation from leaves and petioles of physic nut on the MS medium supplemented with plant growth regulators was also reported by Sujatha and Mukta (1996), Kumar and Reddy (2010) and Sujatha et al (2005).

The combination of 0.1 mg/l TDZ and 300 mg/l proline (7.7%) increased percentage of shoot regeneration (Table 5). Proline was one of the growth additives which enhanced shoot proliferation and elongation in physic nut (Maharana et al., 2012). Proline was reported to support plant growth by both regulating the intracellular osmotic pressure (Bandurska, 1993; Solomon et al., 1994; Khaleda and Al-Forkan, 2006) and scavenging a toxic free radical (Smirnoff and Cumbes, 1989). The growth additive and cytokinin may synergize in promoting cell growth and shoot multiplication (Shetty and Mark, 2004).

The growth additive, such as casein hydrolysate, was reported to enhance shoot regeneration (Mehra and Mehra, 1974; James et al., 1984; Varshney and Johnson, 2010; Cao et al., 1998) by providing plant cell with a source of minerals (nitrogen, calcium, potassium) and vitamins (George, 1993; Khaleda and Al-Forkan, 2006).

However, the adventitious shoots formation was also observed in the medium without casein hydrolysate, a growth additive (Pellegrineschi, 1997). This may be because some explants may have contained high level of protein and amino acid and addition of these nitrogenous compounds is not required for inducing the formation of adventitious shoot (Pellegrineschi, 1997). In this study, the MS medium containing TDZ (0.1 mg/l) and casein hydrolysate (500 mg/l) did not induce adventitious shoot formation from leaf explants. The role of casein hydrolysate, when used in combination with other plant growth regulators, is thus complicated and more research is needed to determine its role in regenerating shoot in different plant genotypes and explant types.

Conclusion

The combination and concentration of cytokinin and auxin affected adventitious shoot regeneration from either callus (derived from young hypocotyl of seedling) or leaf explants (derived from young leaf of seedling) of *J. curcas* (physic nut) in tissue culture.

In Experiment 1.1., TDZ was most effective hormone for shoot regeneration although single supplemented in MS medium.

An increased shoot regeneration from callus in Experiment 1.2 was found in MS medium supplemented with decreased IBA concentration (at 0.01 mg/l and 1.0 mg/l) (compared with treatments in Experiment 1.1) combined with 0.5 mg/l BA. However, the highest value of shoot regeneration was observed in MS medium supplemented with 2.0 mg/l BA and 0.5 mg/l IAA.

In Experiment 2, the combinations of 0.5 mg/l BA and 1.0 mg/l IBA and the combination of 0.1 mg/l TDZ and 300 mg/l proline in MS medium were most effective on shoot regeneration from leaf explants.

Acknowledgements

We are grateful to the Silpakorn University Research and Development Institute (SURDI), Silpakorn University, Thailand for funding. We would also like to thank Faculty of Animal Science and Agricultural Technology for supports during the course of this research.

References

Ahmed, A. B. A., Pallela, R., Rao, A. S., Rao, M. V., and Mat Taha, R. (2011) Optimized conditions for callus induction, plant regeneration and alkaloids accumulation in stem and shoot tip explants of *Phyla nodiflora*. *Spanish Journal of Agricultural Research* 9(4): 1262-1270.

Ahmed, M. R. and Anis, M. (2012) Role of TDZ in the quick regeneration of multiple shoots from nodal explant of *Vitex trifolia* L.-an important medicinal plant. *Applied Biochemistry and Biotechnology* 168(5): 957-966.

Bandurska, H. (1993) *In vitro* and *in vivo* effect of proline on nitrate reductase activity under osmotic stress in barley. *Acta Physiologiae Plantarum* 15: 83-88.

Bhansali, R. (1990) Somatic embryogenesis and regeneration of plantlets in pomegranate. *Annual of Botany* 66: 249-254.

Biradar, S., Waghmare, V., and Pandhure, N. (2012) *In vitro* callus and shoot induction in *Jatropha curcas* (Linn.). *Trends of Life Sciences* 1: 38-41.

Burikam, S. and Kawita, R. (2007) Tissue Culture of *Jatropha* (*Jatropha curcus* L.) *in vitro*. [Online URL: http://rdi.ku.ac.th/kufair50/plant/20_plant/20_plant.html] accessed on June 21, 2014. (Thai version)

Cao, X., Liu, Q., Rowland, L. J., and Hammerschlag, F. A. (1998) GUS expression in blueberry (Vaccinium spp.): factors influencing *Agrobacterium*-mediated gene transfer efficiency. *Plant Cell Reports* 18:266-270.

da Camara Machado, A., Frick, N. S., Kremen, R., Katinger, H., and da Camara Machado, M. L. (1997) Biotechnological approaches to the improvement of *Jatropha curcas*. In *Proceedings of the International Symposium on Jatropha*, pp. 15. Managua, Nicaragua.

Divakara, B. N., Upadhyaya, H. D., Wani, S. P., and Laxmipatha Gowda, C. L. (2009) Biology and genetic improvement of *Jatropha curcas* L.: A review. *Applied Energy*. Doi:10.1016/j.apenerge. 2009.07.013.

Gallo-Meagher, M., English, R. G., and Abouzid, A. (2000) Thidizuron stimulatings shoot regeneration of sugarcane embryogenic callus. *In Vitro Cellular and Developmental Biology - Plant* 36: 37-40.

George, E. F. (1993) Part 1: The technology. In *Plant propagation by tissue culture*, 2nd ed, pp. 285-291. London: Exegetics Ltd.

Guo, B. Abbasi, B. H., Zeb, A., Xu, L. L., and Wei, Y. H. (2011) Thidiazuron: a multi-dimensional plant growth regulator. *African Journal of Biotechnology* 10(45): 8984-9000.

Heller, J. (1996) Physic nut, *Jatropha curcas* L. In *Promoting the conservation and use of underutilized and neglected crops. No 1*, pp 66. Rome: International Plant Genetics Resource Institute.

Huetteman, C.A. and Preece, J. E. (1993) TDZ: a potent cytokinin for woody plant tissue culture. *Plant Cell, Tissue and Organ Culture* 33: 105-119.

Hutchinson, M. J., Senaratna, T., Tsujita, J. M., and Saxena, P. K. (1997) Somatic embryogenesis in liquid cultures of a tetraploid *Alstroemeria*. *Plant Cell, Tissue and Organ Culture* 47: 293-297.

James, D. J., Preece, J. E., and Malhotra, S. B. (1984) Organogenesis in callus derived from stem and leaf tissues of apple and cherry rootstocks. *Plant Cell, Tissue and Organ Culture* 3: 333-341.

Jha, T. B., Mukherjee, P., and Datta, M. M. (2007) Somatic embryogenesis in *Jatropha curcas* Linn an important biofuel plant. *Plant Biotechnology Report* 1: 135-140.

Jose, J., Nimisha, K., Anu, M. A., and Nambisan, P. (2012) Evaluation of somaclonal variation in callus cultures of *Jatropha curcas* maintained on different hormonal combination using RAPD makers. *World Journal of Agricultural Sciences* 8(6): 616-623.

Kaewpoo, M. and Te-chato, S. (2009) Influence of explant types and plant growth regulators on multiple shoot formation from *Jatropha curcas*. *ScienceAsia* 35: 353-357.

Kapil, S. S. and Sharma, V. (2014) *In-vitro* propagation of *Bacopa monneri*: an important medicinal plant. *International Journal of Current Biotechnology* 2(1): 7-10.

Karataş, M., Aasim, M., Çinar, A., and Dogan, M. (2013) Adventitious shoot regeneration from leaf explant of dwarf hygro (*Hygrophila polysperma* (Roxb.) T. Anderso). *The Scienctific World Journal* 2013: 1-7.

Khaleda, L. and Al-Forkan, M. (2006) Stimulatory effects of casein hydrolysate and proline in *in vitro* callus induction and plant regeneration from five deep water rice (*Oryza sativa* L.). *Biotechnology* 5(3): 379-384.

Khurana-Kaul, V., Kachhwaha, S., and Kothari, S. L. (2010) Direct shoot regeneration from leaf explants of *Jatropha curcas* in response to thidiazuron and high copper contents in the medium. *Biologia Plantarum* 54(2): 369-372.

Kumar, N. and Reddy, M. P. (2010) Plant regeneration through the direct induction of shoot buds from petiole explants of *Jatropha curcas*: a biofuel plant. *Annals of Applied Biology* 156(3): 367-375.

Li, M., Li, H., Jiang, H., Pan, X., and Wu, G. (2007) Extablishment of an *Agrobacterium*-mediated cotyledon disc transformation method for *Jatropha curcas*. *Plant Cell, Tissue and Organ Culture* 92: 173-181.

Lin, H. S., van der Toorn, C., Raemakers, K. J. J. M., Visser, R. G. F., De Jeu, M. J., and Jacobsen, E. (2000) Development of a plant regeneration system based on friable embryogenic callus in the ornamental *Alstroemeria*. *Plant Cell Reports* 19: 529-534.

Lu, C. Y. (1993) The use of TDZ in tissue culture. *In Vitro Cellular and Developmental Biology - Plant* 29P: 92-96.

Maharana, S. B., Mahato, V., Behera, M., Mishra, R. R., and Panigrahi, J. (2012) *In vitro* regeneration from node and leaf explants of *Jatropha curcas* L. and evaluation of genetic fidelity through RAPD markers. *Indian Journal of Biotechnology* 11: 280-287.

Mazumdar, P., Basu, A., Paul, A., Mahanta, C., and Sahoo, L. (2010) Age and orientation of the cotyledonary leaf explants determine the efficiency of de novo plant regeneration and *Agrobacterium tumefaciences*-mediated transformation in *Jatropha curcas* L. *South African Journal of Botany* 76: 337-344.

Mehra, A. and Mehra, P. N. (1974) Organogenesis and plantlet formation *in vitro* in almond. *Botanical Gazette* 135 (1): 61-73.

Murashige, T. and F. Skoog. (1962) A revised medium for rapid growth and bioassays with tobacco cultures. *Plant Physiology* 15: 473-497.

Murthy, B. N. S., Murch, S. J., and Saxena, P. K. (1998) Review: Thidiazuron: a potential regulator of *in vitro* plant morphogenesis. *In Vitro Cellular and Developmental Biology - Plant* 34: 267-275.

Nunes, C. F., dos Santos, D. N., Pasqual, M., Valente, T. T., de Oliveira, A. C. L., Alves, E., and Setotaw, T. A. (2013) Morphogenesis and regeneration of adventitious shoots in *Jatropha*

curcas L. *Australia Journal of Crop Science* 7(10): 1511-1519.

Palai, S. K., Rout, G. R., and Das, P. (1997) Micropropagation of ginger (*Zingiber officinale* Rosc.) – interaction of growth regulators and culture conditions. In *Proceedings of the national semimar on biotechnology of species and aromatic plants*, pp. 20-24. Calicut, India.

Pan, J., Fu, Q., and Zeng-Fu, X. U. (2010) *Agrobacterium tumefaciens*-mediated transformation of biofuel plant *J. curcas* using kanamycin selection. *African Journal of Biotechnology* 9: 6477-6481.

Pellegrineschi, A. (1997) *In vitro* plant regeneration via organogenesis of cowpea [*Vigna unguiculata* (L.) Walp.]. *Plant Cell Reports* 17: 89-95.

Preece, J. E. and Imel, M. R. (1991) Plant regeneration from leaf explants of Rhododendron 'P. J. M. Hybrids'. *Scientia Horticulturae* 48(1-2): 159-170.

Premkumar, G., Sankaranarayanan, R., Jeeva, S., and Rajarathinam, K. (2011) Cytokinin induced shoot regeneration and flowering of *Scoparia dulcis* L. (Scrophulariaceae) – an ethnomedicinal herb. *Asia Pacific Journal of Tropical Biomedicine* 1(3): 169-172.

Raghu, A. V., Geetha, S. P., Martin, G., Balachandran, I., and Ravindran, P. N. (2006) Direct organogenesis from leaf explants of *Embelia ribes* Burm. – a vulnerable medicinal plant. *Journal of Forest Research* 11(1): 57-60.

Rajore, S. and Batra, A. (2005) Efficient plant regeneration via shoot tip explant in *Jatropha curcas*. *Journal of Plant Biochemistry and Biotechnology* 14: 73-75.

Sardana, J., Batra, A., and Ali, D. J. (2000) An expeditious method for regeneration of somatic embryos in *Jatropha curcas* L. Phytomophology 50: 239-242.

Sharma, V., Gupta, S. K., and Dhiman, M. (2012) Regeneration of plants from nodal and intermodal segment cultures of *Ephedra gerardiana* using Thidiazuron. *Plant Tissue Culture and Biotechnology* 22(2): 153-161.

Shetty, K. and Mark, W. (2004) A model for the role of the proline-linked pentose phosphate pathway in phenolic phytochemical biosynthesis and mechanism of action for human health and environmental applications. *Asia Pacific Journal of Clinical Nutrition* 13: 1-24.

Singh, A., Reddy, M. P., Chikara, J., and Singh, S. (2010) A simple regeneration protocol from stem explants of *Jatropha curcas*. *Industrial Crops and Products* 31: 209-213.

Smirnoff, N. and Cumbes, Q. J. (1989) Hydroxyl radical scavenging activity of compatible solutes. *Phytochemistry* 28: 1057-1060.

Solomon, A., Beer, S., Waisel, Y., Jores, G. P., and Paleg, L. G. (1994) Effects of NaCl on the carboxylating activity of Rubiso from *Tamarix jordanis* in the presence and absence of proline-related compatible solutes. *Physiologia Plantarum* 90: 198-204.

Subroto, A. P., Utomo, C., Darmawan, C., Hendroko, R., and Liwang, T. (2014) Tissue culture media optimization and genetic transformation of *Jatropha curcas* genotype *Jatromas* cotyledon explants. *Energy Procedia* 47: 15-20.

Sujatha, M. and Dhingra, M. (1993) Rapid plant regeneration from various explants of *Jatropha integerrima*. *Plant Cell, Tissue and Organ Culture* 35: 293-296.

Sujatha, M. and Mukta, N. (1996) Morphogenesis and plant regeneration from tissue cultures of *Jatropha curcas*. *Plant Cell, Tissue and Organ Culture (Historical Archive)* 44: 135-141.

Sujatha, M., Makkar, H. P. S., and Becker, K. (2005) Shoot bud proliferation from axillary nodes and leaf sections of non-toxic *J. curcas* L.

Plant Growth Regulation 47: 83-90.

Sujatha, M. and Reddy, T. P. (1998) Differential cytokinin effects on the stimulation of *in vitro* shoot proliferation from meristematic explants of castor (*Ricinus communis* L.). Plant Cell Report 17: 561-566.

Thepsamran, N., Thepsithar, C., and Thongpukdee, A. (2008) *In vitro* induction of shoots and roots from *J. curcas* L. explants. *Journal of Horticultural Science and Biotechnology* 83: 106-112.

Varshney, A. and Johnson, T. S. (2010) Efficient plant regeneration from immature embryo cultures of *Jatropha curcas*, a biodiesel plant. *Plant Biotechnology Report* 4(2): 139-148.

Verma, S. K., Yücesan, B. B., Şahın, G., Gürel, S., and Gürel, E. (2011) Direct shoot regeneration from leaf explants of *Digitalis lamarckii*, an endemic medicinal species. *Turkish Journal of Botany* 35: 689-695.

Wei, Q., Lu, W.-D. Liao, Y., Pan, S. L. Xu, Y., Tang, L., and Chen, F. (2004) Plant regeneration from epicotyl explant of *Jatropha curcas*. *Journal of Plant Physiology and Molecular Biology* 30: 475-478.

Wirasutisna, K. R. and Artri, E. (2011) Reducing of phorbol ester content in callus cultures of physic nut (*Jatropha curcas* L.) using manganese chloride and n-ethylmaleimid. *Pharmacognosy Journal* 3(20): 42-46. Doi: 10.5530/pj.2011.20.9

Winkler, E., Gübitz, G. M., Foidl, N., Staubmann, R., and Steiner, W. (1997) Use of enzymes for oil extraction from *Jatropha curcas* seeds. In *Proceeding "Jatropha curcas"* (Gübitz, G. M., Mittelbach, M., and Trabi, M. eds.) Managua, Nicaragua.

Yeoman, M. M. and Yeoman, C. L. (1996) Manipulating secondary metabolism in cultural plant cells. *New Phytologist* 134: 553-569.

Zalewska, M., Lema-Rumińska, J., Miler, N., Gruszka, M., and Dąbal, W. (2011) Induction of adventitious shoot regeneration in chrysanthemum as affected by the season. *In Vitro Cellular and Development Biology - Plant* 47: 375-378.