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In vitro PROPAGATION OF *Adenosma indianum* (Lour.) Merr.

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ABSTRACT

This paper presents the results of *in vitro* propagation of *Adenosma indianum* (Lour.) Merr. The obtained results indicated that shoot regeneration from stem explant achieved on MS basal medium supplemented with 0.5 mg/L BAP after 4 weeks of culture, 46.7% of samples regenerating shoots with an average value of 1.8 shoots per sample. The MS medium containing 0.30 mg/L NAA was suitable for callus induction from leaves and containing 0.50 mg/L BAP and 0.10 mg/L NAA had a highest ability to produce buds from callus, with a ratio of 43.52%, and 3.43 shoots/callus. The multiplication coefficient was greatest on MS medium consist of 0.5 mg/L BAP and 0.3 mg/L IBA, average number of shoots per explant was 10.03 after 6 weeks of culture. The MS medium supplemented with 0.25 mg/L NAA was optimal for rooting (average value of 7.17 roots per shoot). The plantlets were acclimatized and transplanted successfully in the coconut coir pots, the plantlet survival rate was 91.67% after two months of cultivation. The *in vitro* propagation procedure of *A. indianum* (Lour.) Merr was developed successfully, shoots can be regenerated directly from axillary bud or callus and *in vitro* plantlets grow well in greenhouse.

Keywords: *Adenosma indianum*; callus induction; medicinal plant; shoot regeneration; shoot tip; stem.

ABBREVIATIONS

BAP: 6-Benzylaminopurine; IBA: Indole-3-butyric acid; NAA: Naphthalene acetic acid; MS: Murashige and Skoog; PGRs: Plant growth regulators.

INTRODUCTION

Adenosma is a genus of aromatic and ornamental perennial plants of Scrophulariaceae in the South and South-East Asia and Oceania, which include about 15 species [1]. *Adenosma indianum* (Lour.) Merr (synonym *Adenosma indiana, Adenosma capitatum, Manulea indiana…*) is a valuable medicinal plant used in traditional Chinese as well as Vietnamese medicine. Ji and Pu (1985) reported that the main components in the *A. indianum* are α-pinene, β-pinene, limonene, p-cymene, 1,4-cineol, linalool, fenchone, o-methylanisole and δ-guaiene [2]. They play an important role in pharmaceutical for treatment of jaundice in viral hepatitis, oliguria, biliuria, fever, ophthalmalgia, vertigo and dyspepsia [2-4].

Adenosma indianum (Lour.) Merr can be obtained from wild sprouts in midland provinces of

Vietnam. The biomass of these species has been decreased recently due to high demand as well as the urbanization. The natural regenerative capacity of *A. indianum* is very low. In order to propagate *A. indianum*, some people have been experimenting in growing them using traditional plant propagation methods such as seeds sowing, grafting, etc. However, it often results in low survival ratio of seedlings and disease infection. Using plant tissue culture techniques can help resolving the difficulties facing such traditional methods.

There have been a several reports on *A. indianum* (Lour.) Merr, however, most of researches focused on distribution [5], analysis of biological characteristics and natural regenerative capacity, chemical composition and biological activity [2, 6], volatile components [7], chemical composition of betulinic acid, antimicrobial properties of essential oils of *A. indianum* (Lour.) Merr. [8],… and only a little of works presented the results of *in vitro* propagation of *Adenosma glutinosum* (L.) Druce. For instances, Liu et al. (2012) successfully propagated of *A. glutinosum* (L.) Druce by *in vitro* plant cell tissue culture technique [9]. Tu et al. (2012) also reported a highly efficient method for *in vitro* adventitious shoot regeneration of *A. glutinosum* (L.) Druce using leaf samples [1]. However, there has been no publication related to *in vitro* propagation of *A. indianum* (Lour.) Merr. This paper presented our results of *in vitro* propagation of *A. indianum* (Lour.) Merr.).

MATERIALS AND METHODS

Materials

Our materials were *Adenosma indianum* (Lour.) Merr, a species in the genus *Adenosma* (Scrophulariaceae family). Our primary culture materials were shoot tips and node stems of naturally grown *A. indianum* taken from Thua Thien Hue Province (Fig. 1a), Vietnam. The research sample was provided by Hue University of Agriculture and Forestry.

Preparation for Culture and Explant Sterilization

The stem (0.5 cm) and shoot tip (0.5 cm) of a healthy *A. indianum* were rinsed three times thoroughly in dilute soap liquid and then several times under tap water. The explant was subsequently washed 5 times in sterile distilled water before being put into a transplant cabinet. In the cabinet, the explant was preliminarily sterilized with 70% alcohol in 30 seconds and surface sterilized in 0.1% $HgCl_2$ solution for 6 mins. After that, the explant was re-rinsed through sterile distilled water for 6-7 times before being cultured onto the medium.

All the experiments were conducted at the temperature of 25±2ºC, the light intensity of 2000-3000 lux and the lighting time of 16 hours/day.

Shoot Regeneration

The shoot tips and shoot nodal stem segments with axillary bud (0.5 cm) were implanted on MS (Murashige and Skoog, 1962) basal medium [10] supplemented with 3% sucrose, 0.8% agar and additional plant growth regulators (PGR) including BAP (0.25-1.00 mg/L) or KIN (0.25-1.00 mg/L) in order to monitor the regenerative capacity of *A. indianum* (Lour.) Merr. The assessment parameters include the number of shoots and height of shoots obtained after 4 weeks of culture.

Callus Induction and Adventitious Buds from Callus

Explants (leaf, petiole and stem segments) were cultured on MS medium supplemented with IBA (0.10-0.50 mg/L) or NAA (0.10-0.50 mg/L) for inducing callus. Leaf-derived callus were cultured on MS medium supplemented with 0.5 mg/L BAP and 0.10-0.50 mg/L NAA for adventitious buds [1].

The percentage of callus induction, the percentage of adventitious bud induction and the average number of adventitious buds per callus were observed after 8 weeks of cultivation.

Shoot Multiplication

The apical buds (2-3 cm in length) from *in vitro* shoot (or adventitious buds from callus) were cultured on MS basal medium supplemented with BAP (0.25-1.00 mg/L), KIN (0.25-1.00 mg/L) alone or in a combination with NAA (0.1-0.5

mg/L) or IBA (0.1-0.5 mg/L) in order to test the *in vitro* shoot multiplication ability. The assessment parameters include the number of shoots, height of shoots, number of leaves/bud obtained after 6 weeks of culture.

Rooting

In vitro shoots (2-3 cm in length) were cultured on MS basal medium supplemented with NAA (0.25-1.00 mg/L) or IBA (0.25-1.00 mg/L) in order to monitor the rooting capacity. The assessment parameters include the number of roots/shoot and height of roots after 6 weeks of culture.

Transplanting to the Greenhouse

After 6 weeks cultured on rooting medium, *in vitro* plantlets was 4-6 cm in height, well-developed roots were transferred from culture room to greenhouse for acclimatization in 1 week. After that, plantlets were removed from the cultural bags and transplanted into plastic pots containing pre-watered substrates (sand, coir, soil and a mixture of sand and soil in 1:1 ratio). *In vitro* plantlets were cleaned of rooting medium, soaked in 3% Citizen 75WP fungicide (Tricylazole 75%, additives 25%) in 10 minutes and subsequently cleaned carefully. After getting their roots clipped, they were planted on growing substrates. The survival ratio, shoot height, number of roots and length of roots were observed after 4 weeks.

Statistical Analysis

Each experiment was repeated 3 times, 15 samples were observed in each formula. The data were analyzed through Duncan's test by using SPSS 19.0 software with p=0.05.

RESULTS AND DISCUSSION

Shoot Regeneration from Stems and Shoot Tips

The sterilized samples were cultured on MS medium supplemented with KIN or BAP with the concentration ranged from 0.25 to 1.0 mg/L. On the regeneration medium, the samples of axillary shoots didn't regenerate more buds but increased the shoot height only. The influences of KIN and BAP concentration on the shoot regenerative capacity after 4 weeks of monitoring were shown in Table 1.

On the MS mediums supplemented with KIN, only the concentration value of 0.25 and 0.5 mg/L KIN stimulated the *in vitro* growth and shoot multiplication. The number of shoots/sample obtained were 1.60 and 1.20 shoots/sample respectively. The shoot height reached the average of 1.9-2.12 cm, which not statistically significant. Obtained shoots were quite thick and healthy with thick leaves and green color; each shoot regenerated two small axillary shoots.

On the medium containing 0.5 mg/L BAP, there were 46.7% of stem samples regenerating shoots, with an average value of 1.8 shoots per sample and shoot height was 2.26 cm. Obtained shoots had green color and small leaves; each shoot bud regenerated two small axillary shoots. (Fig. 1b). The regenerative rate decreased to 13.33% when the concentration of BAP increased to 0.75 mg/L, shoots were 1.27 cm in height.

Table 1. The influences of PGRs on the shoot regenerative capacity after 4 weeks

PGRs (mg/L)		Regeneration rate (%)	Number of shoots per sample	Shoot height (cm)
KIN	BAP			
0.00	0.00	0	0	0
0.25	-	30	1.60^{ab}	2.12^{a}
0.50	-	13.33	1.20^{b}	1.98^{a}
0.75	-	0	0	0
1.00	-	0	0	0
-	0.25	20.00	1.11^{b}	1.74^{a}
-	**0.50**	**46.70**	**1.80^{a}**	**2.26^{a}**
-	0.75	13.33	1.27^{b}	1.95^{a}
-	1.00	0	0	0

Different letters on the same column indicate a statistically significant difference of the sample means with the p-value = 0.05 (Duncan's test). This note were used for all tables

The shoot regenerative ratio of *A. indianum* was lower than that of species of Scrophulariaceae family. Only 46.7% of stems regenerated shoots, with an average value of 1.8 shoots/samples and 2.26 cm in height. *In vitro* propagation of genus *Scoparia* and *Bacopa* species, Scrophulariaceae family resulted in much higher regenerative ratio. Shoot tips and nodal explants (1.0-1.5 cm in length) of *Scoparia dulcis* L were induced better on the MS medium supplemented with 1.5 mg/L BAP and 0.5 mg/L IAA, with 94% of samples and 12 shoots/sample [11]. 100% of stems (1.0-1.5 cm in length) of *Bacopa monnieri* (L.) Penell regenerated shoots on MS medium supplemented with 3.0 mg/L BA with 6.5 shoots/sample and 3.69 cm in height [12]. The *in vitro* propagation research of *B. monnieri* (L.) Wettst showed that 100% regenerated samples with 92.4 shoots/samples were obtained from the mature leaf samples on the MS basal medium supplemented with 1.5 mg/L BAP [13].

Callus Induction

Explants (leaf, petiole and stem segments) was inoculated on MS medium containing auxin group plant growth regulators (IBA and NAA with concentration of 0.10-0.50 mg/L) to explored the ability of callus induction. The results presented in Table 2 shown that calli were appeared in all test media after 3 weeks of induction.

For IBA, leaf samples had the highest callus induction ratio (59.72%) at IBA concentration of 0.30 mg/L, petioles were 66.67% at 0.30 mg/L and stem segments were also 66.67% at a concentration of 0.50 mg/L.

Compared to IBA, NAA was better PGR for callus induction of *A. indianum.* For leaf samples, the ratio of callus formation was up to 80.55% (0.30 mg/L NAA) (Fig. 1c), petioles were 58.93% and stem segments were 76.39% (0.50 mg/L NAA).

Thus, the MS medium supplemented with 0.30 mg/L NAA was suitable for callus induction from leaves and stem segments, the MS medium containing 0.3 mg/L IBA was suitable for callus formation from petiole.

Shoot Regeneration from Callus

In this study, we found that leaves were suitable for callus production, so we used leaf-derived callus to regenerated buds *in vitro*. Calli were formed during *in vitro* culture and were cultured on MS medium supplemented with 0.50 mg/L BAP and NAA (concentration of 0.10-0.50 mg/L) to investigated the regeneration of shoots from callus.

The results presented in Table 3 shown that callus in all experiments were capable of regenerating into shoots. The MS medium containing 0.50 mg/L BAP and 0.10 mg/L NAA had a higher ability to produce buds from callus than others, with a ratio of 43.52%, and number of shoots/callus were 3.43. Shoots were good growth, and leaves were dark green (Fig. 1d). MS medium with higher NAA concentrations had lower shoot regeneration, in these media, the growth rate of shoots was poor and leaves had light green color.

Table 2. Effects of IBA and NAA concentration on callus induction

PRGs (mg/L)		Callus induction (%)		
IBA (mg/L)	NAA	Leaf	Petioles	Stem segments
0.10	-	33.33^{e}	50.00^{a}	41.67^{bc}
0.20	-	44.44^{de}	63.89^{a}	44.44^{bc}
0.30	-	59.72^{bcd}	66.67^{a}	35.00^{c}
0.40	-	56.94^{cd}	61.11^{a}	38.89^{bc}
0.50	-	51.39^{de}	58.33^{a}	66.67^{ab}
-	0.10	72.22^{abc}	45.44^{a}	38.08^{c}
-	0.20	74.45^{abc}	48.61^{a}	44.44^{bc}
-	0.30	80.55^{a}	48.61^{a}	51.11^{abc}
-	0.40	78.89^{ab}	51.81^{a}	56.94^{abc}
-	0.50	49.44^{de}	58.93^{a}	76.39^{a}

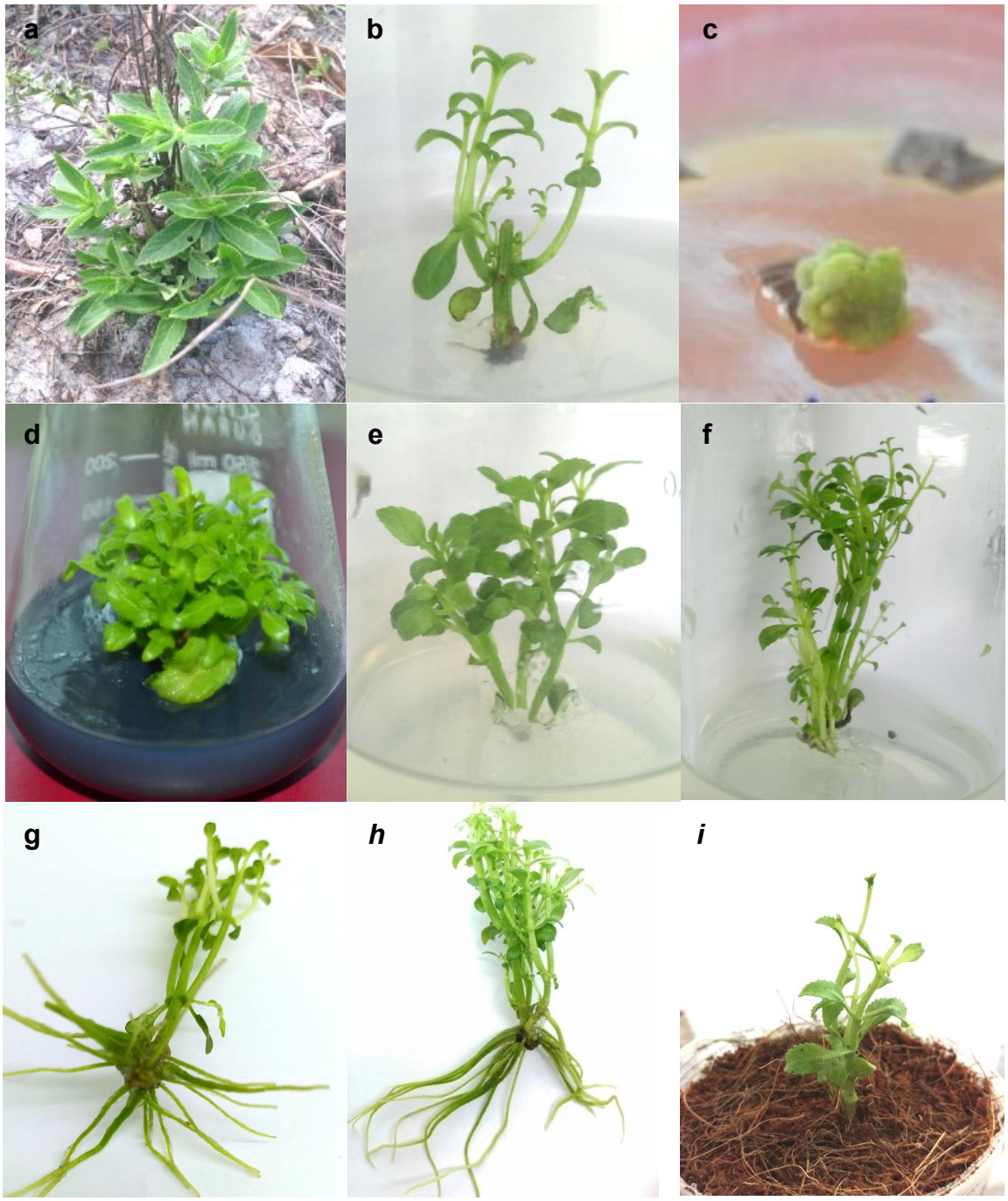

Fig. 1. *In vitro* propagation of *A. indianum*. Natural *A. indianum* (a), Shoot regeneration on MS medium supplemented with 0.50 mg/L BAP (b), Leaf-derived callus (c), Shoot regeneration on MS medium containing 0.50 mg/l BAP and 0.10 mg/l NAA (d), Shoot propagation on MS medium supplemented with 0.5 mg/L BAP (e) or 0,5 mg/L BAP and 0,3 mg/L IBA (f), Roots on MS medium supplemented with 0,5 mg/L NAA (g) or 0.25 mg/L IBA (h), *In vitro* plants after 2 weeks planted on coir substrate (i)

Table 3. Effects of PGRs on shoot regeneration from callus

PRGs (mg/l)		Shoot regeneration ability	
BAP	NAA	Shoot regeneration ratio (%)	Number of shoots/callus
0.50	0.10	43.52[a]	3.43[a]
0.50	0.20	22.22[ab]	1.83[ab]
0.50	0.30	13.89[b]	1.60[ab]
0.50	0.50	6.48[b]	0.49[b]

In in report of *A. glutinosum* propagation, Tu et al. (2012) found that MS medium supplementary with 0.50 mg/L BAP and 0.01 mg/L NAA obtained 7.2 buds/callus, higher than our study [1]. Thus, it can be seen that in the same culture conditions, the *in vitro* propagation capacity of *A. glutinosum* is higher than that of *A. indianum*. According to Majumder et al. (2011), the callus of *Scoparia dulcis* Linn had the highest regenerative rate on MS medium supplemented with 1.5 mg/L BAP and 0.5 mg/L IAA, reaching 28.1 shoots/callus [14].

Shoot Multiplication

In vitro shoots were used as culture materials for the shoot multiplication process. We examined the individual effects of cytokinine (BAP, KIN) and the combination of cytokinine and auxin (BAP and NAA) on the rapid propagation of *in vitro* shoots. The individual effects of BAP, KIP on the shoot multiplication after 6 weeks of culture were shown in the Table 4.

In the MS medium containing KIN, the highest number of shoots was obtained at the concentration of 0.25 mg/L (2.62 shoots/sample, 2.45 cm in height with 3.78 leaves/shoot). Shoots were good quality and healthy with thick body, wide leaves and dark green color. The number of shoots decreased when the concentration of KIN increased from 0.5 to 1.0 mg/L. MS basal medium supplemented with 0.25-1.0 mg/L BAP stimulated more *in vitro* shoot than that of KIN. On MS medium containing 0.5 mg/L BAP, the number of shoots reached 3.85 shoots/sample (2.33 cm in height and 3.44 leaves) (Fig. 1e). When the BAP concentration increased from 0.75 to 1.0 mg/L, the number of obtained shoots gradually decreased. On MS medium supplemented with BAP, the shoots produced had thick, thick leaves and dark green color.

Therefore, the best formula for shoot multiplication was MS medium supplemented with BAP 0.5 mg/L. From this results, we continued estimated the effects of 0.5 mg/L BAP combined with NAA or IBA at various concentrations on the shoot multiplication capacity of *A. indianum*. The effects of BAP combined with NAA on the shoot multiplication capacity after 6 weeks of culture were shown in Table 5.

The MS medium consist of 0.5 mg/L BAP and 0.2 mg/L NAA had the best effect on shoot multiplication of *A. indianum*, the of shoots/sample were highest (4.54 shoots/sample). Obtained shoots were of high quality with thick shoots, thick leaves and dark green color. Shoots grew fastest on the MS medium supplemented with 0.5 mg/L BAP and 0.3 mg/L NAA (shoot height 3.35 cm with 4.80 leaves/shoot). The number of shoots/sample decreased when the NAA concentration increased. The number of shoots obtained on MS medium supplemented with 0.5 mg/L BAP and 0.5 mg/L NAA was relatively low (1.40 shoots/samples), lower than of control. Shoots were poor quality, small stems, thin leaves and light green color. On this medium, there was about 50% of shoots had roots (approximate 10-15 roots/shoot). Roots grew healthily, had light green color and formed into cluster.

Besides NAA, other auxin IBA had the good effect on *in vitro* shoot multiplication when it was combined with cytokinine, so we also estimated the effects of 0,5 mg/L BAP combined with IBA on the shoot multiplication capacity after 6 weeks. The MS medium supplemented with 0.5 mg/L BAP and IBA (0.1-0.5 mg/L) stimulated the *in vitro* shoot multiplication capacity quite well (Table 6).

Table 4. Effects of KIN and BAP on the shoot multiplication after 6 weeks of culture

KIN (mg/L)	BAP (mg/L)	Number of shoots/sample	Shoot height (cm)	Number of leaves/shoot
0.00	-	1.33^{d}	1.79^{b}	2.19^{cd}
0.25	**-**	**2.62^{bc}**	**2.45^{a}**	**3.78^{a}**
0.50	-	1.78^{d}	2.31^{ab}	3.06^{b}
0.75	-	1.34^{d}	1.60^{b}	2.57^{bc}
1.00	-	0.58^{e}	0.86^{c}	1.89^{d}
-	0.25	2.43^{c}	1.96^{b}	2.87^{b}
-	**0.50**	**3.85^{a}**	**2.53^{a}**	**3.44^{ab}**
-	0.75	3.08^{b}	2.07^{ab}	2.13^{cd}
-	1.00	2.25^{c}	1.36^{bc}	1.32^{e}

Table 5. Effects of 0.5 mg/L BAP combined with NAA on the shoot multiplication capacity after 6 weeks of culture

NAA (mg/L)	Number of shoots/sample	Shoot height (cm)	Number of leaves/shoot
0.0	2.42^{c}	2.32^{d}	2.23^{c}
0.1	2.92^{b}	2.39^{d}	4.00^{bc}
0.2	**4.54^{a}**	**2.56^{cd}**	**4.49^{ab}**
0.3	3.40^{b}	3.35^{a}	4.80^{a}
0.4	2.44^{c}	3.19^{ab}	4.52^{ab}
0.5	1.40^{d}	2.90^{c}	3.80^{c}

Table 6. Effects of 0.5 mg/L BAP combined with IBA on the shoot multiplication capacity

IBA (mg/L)	Number of shoots/sample	Shoot height (cm)	Number of leaves/shoot
0.0	2.42^{f}	2.32^{d}	2.23^{c}
0.1	4.42^{e}	2.97^{d}	3.84^{bc}
0.2	6.14^{d}	4.34^{c}	4.40^{ab}
0.3	**10.03^{a}**	**5.14^{b}**	**4.44^{ab}**
0.4	8.90^{b}	5.59^{a}	4.88^{a}
0.5	7.32^{c}	4.53^{c}	3.92^{bc}

MS mediums including 0.5 mg/L BAP and 0.3 mg/L IBA were suitable for shoot multiplication, the highest number of shoots/sample reached 10.03 shoots/sample. The shoots formed into clusters and grew rapidly in height, with thin shoots; small leaves, dark green color; the quality of shoots was quite high (Fig. 1f). In this experiment, the control (MS medium supplemented with 0.5 mg/L BAP) reached only 2.42 shoots/sample, which was much lower than on medium supplemented with 0.5 mg/L BAP combined with 0.3 mg/L IBA. However, shoots grew fastest on medium consist of 0.5 mg/L BAP and 0.4 mg/L IBA. When the concentration of IBA increased, the number of shoots/sample slowly decreased. The total number of shoots produced per sample was 7.32 shoots on medium supplemented with 0.5 mg/L BAP combined with 0.5 mg/L IBA. Obtained shoots were of good quality with small stems, small leaves and dark green color. On the MS medium supplemented with 0.5 mg/L BAP and 0.4-0.5 mg/L IBA, roots were produced on several shoots, the number of roots per shoot were 1-2 roots/shoot. Obtained roots were long, grew rapidly and didn't branch out.

According to Liu et al. 2010, MS medium supplemented with 0.25 mg/L BA and 0.5 mg/L IBA allowed the growth of adventitious shoots on shoot tips of *Adenosma glutinosum* (L.) Druce [9]. Tu et al. 2012 studied the shoot regeneration of *Adenosma glutinosum* on the medium supplemented with the combination of 0.5 mg/L

BAP and NAA 0.1 mg/L, which resulted in the highest rate of adventitious shoots forming from callus, nearly 100% and reaching 7.2 shoots/samples [1].

BAP and kinetin have played an important role in the shoot multiplication of various plants. Supplementing MS medium with BAP gave better results of shoot multiplication than with kinetin [15, 16]. According to Jain et al. (2010), MS medium supplemented with 0.5 mg/L BAP was suitable for shoot multiplication of *Bacopa minnieri* (reaching 3.4 shoots/sample) [17]. In our study, using the combination of BAP and NAA or IBA can help increasing the number of shoots/sample compared to only using BAP, in which the medium supplemented with the combination of BAP and IBA gave much higher number of shoots/sample than those of BAP and NAA. These results were similar to those obtained from studies on some plants belonging to the Scrophulariaceae family such as *Bacopa monnieri*. Vijayakumar et al. (2010) reported that MS medium containing 1.5 mg/L BAP was suitable for *in vitro* shoot multiplication through shoot tip cultures of *B. monnieri* [16]. According to Asha et al. (2013), 2 mg/L BAP was appropriate for shoot multiplication of *B. monnieri*, reaching 17 shoots/sample [18].

Root Formation

In vitro shoots obtained from above experiments (2-3 cm in length with 2-4 branches) were sprouted separately and cultured on root induction medium with plant growth regulators of auxin group (NAA, IBA) in order to examined the rooting capacity. The results of the effects of PGRs on the formation and development of roots were assessed by the number of roots produced from *in vitro* shoots, the root length after 6 weeks of culture were shown in Table 7.

The results presented in the Table 7 indicated that medium supplemented with NAA or IBA (0.25-1.00 mg/L) stimulated the rooting of *A. indianum*.

Poor rooting and root growth capacity were observed in the control medium (2.37 roots/sample, root length 3.03 cm). The obtained roots were thin, crunchy and fragile. For NAA, the highest number of roots produced were obtained from medium supplemented with 0.5 mg/L NAA, which was 7.96 roots/sample with the length of 5.76 cm. Obtained roots were small and had light green color; the roots were of high quality. The rooting and root growth capacity decreased when the concentration of NAA increased from 0.75 mg/L to 1.00 mg/L. Poor rooting and root growth capacity were observed from medium supplemented with 1.00 mg/L NAA, with the number of roots/shoot only reached 5.70 roots.

The results presented on the Table 8 also showed that IBA (0.25-1.00 mg/L) stimulated the rooting of *in vitro* shoots better than that of NAA. On the medium containing 0.25 mg/L IBA, *in vitro* shoots generated roots rapidly, reaching 14.8 shoots/sample, much higher than of control. Also, the largest average root length (6.35 cm) was observed from medium supplemented with 0.25mg/L IBA as well. The obtained roots were good quality, small and light green color. Increasing the concentration of IBA from 0.5 mg/L to 1.0 mg/L decreased the number of roots. The MS medium supplemented with 1.0 mg/L IBA resulted in poor rooting capacity, the number of roots per shoot was only 6.86.

Table 7. Influences of NAA and IBA on the rooting capacity of *in vitro* shoots after 6 weeks of culture

NAA (mg/L)	IBA (mg/L)	Number of roots/shoot	Root length (cm)
0.00		2.37^{e}	3.03^{d}
0.25		5.98^{d}	4.55^{c}
0.50		**7.96^{cd}**	**5.76^{ab}**
0.75		6.72^{d}	5.01^{bc}
1.00		5.70^{d}	4.27^{cd}
	0.25	**14.8^{a}**	**6.35^{a}**
	0.50	13.42^{b}	5.85^{ab}
	0.75	10.18^{c}	5.37^{bc}
	1.00	6.86^{d}	5.07^{c}

Table 8. Survival ratio and growth of *A. indianum* on the different substrates

Substrate	Survival ratio after 1 month (%)	Plantlet height (cm)	Roots/plantlet	Root length (cm)
Sand	77.78	7.63[a]	6.44[b]	2.40[ab]
Soil	0.00	-	-	-
Coir	91.67	7.89[a]	9.56[a]	2.78[a]
Sand : Soil	88.89	7.33[a]	7.00[b]	2.07[b]

Therefore, the MS medium containing 0.25 mg/L IBA was appropriate for the *in vitro* rooting of *A. indianum*. These results were similar to those of some studies on *in vitro* rooting of plants belonging to Scrophulariaceae family. According to Jain et al. (2014), medium supplemented with 0.25 mg/L BAP was suitable for shoots to produce more roots, reaching 3,5 roots per shoot after 4 weeks of culture of *B. monnieri* [17]. Kumari et al. (2014) reported that MS medium with 0.25 mg/L IBA was appropriate for rooting capacity of *B. monnieri* with 12 roots/shoot (5.6 cm in length) after 4 weeks of culture [15].

Transplanting to the Greenhouse

In the stage of transplanting into the field, the effects of the substrate on the growth of *A. indianum* was monitored. The *in vitro* plantlet (5.0-7.0 cm) after hardening were planted on different substrates, including soil, sand, coir and a mixture of sand and soil in ratio of 1:1.

The survival ratio of *in vitro* plantlets in different substrates reached from 0.00 (soil) to 91.67% (coir) after 1 month of planting (Table 8). On the soil, the plantlets were dead maybe due to high water retention, poor air permeability and water logging. For other substrates, higher porosity and lower water retention make plantlets grow better. In the natural condition, *A. indianum* is a well-grown tree on sandy, which have low water retention, so it is not suitable for good hold water soil types.

The survival ratio of plantlets in this study were similar to the results of *in vitro* culture of several plants belonging to Scrophulariaceae family. The survival ratio of *in vitro Bacopa monnieri* reached from 70 to 80% [17], 95-100% in sand and soil substrates (3:1 ratio) after 3 weeks [18], 90% after 45 days of planting [16] or 82% of *in vitro* plants survived under artificial conditions and 72% of plants under field conditions [15]. *In vitro Scoparia dulcis* had the survival ratio of 85% when transferred to pots containing soil and compost (1:1 ratio) [11].

CONCLUSION

We have succeeded in developing a procedure to propagate *A. indianum* (Lour.) Merr *in vitro*. Shoot were regenerated from stem on MS basal medium supplemented with 0.5 mg/L BAP after 4 weeks of culture. The MS medium containing 0.30 mg/L NAA and 0.50 mg/L BAP plus 0.10 mg/L NAA was suitable for callus induction from leaves and buds formation from callus, respectively. The MS medium consist of 0.5 mg/L BAP plus 0.3 mg/L IBA was good for shoot multiplication and supplemented with 0.25 mg/L NAA was optimal for rooting. The plantlets were transplanted successfully in the coconut coir substrate with the survival rate was 91.67% after two months.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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