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Shoot Tip Culture of *Nepenthes albomarginata* Lobb ex Lindl. In Vitro

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ABSTRAK

Kultur Pucuk Tanaman *Nepenthes albomarginata* Lobb ex Lindl. secara *In Vitro*. *N. albomarginata* adalah kantong semar kerah putih (*white collared pitcher plant*), salah satu tanaman pemakan serangga yang sangat menarik sebagai tanaman hias. Tanaman ini terancam punah karena pengambilan dan kerusakan habitatnya. Penelitian perbanyakan secara *in vitro* dilakukan dengan menggunakan pucuk tanaman *N. albomarginata* pada media formulasi setengah Murashige and Skoog (1/2 MS) dengan tambahan zat pengatur tumbuh 6-benzyladenine (BA) 1 mg l^{-1} dengan atau tanpa kombinasi dengan á-naphthalene acetic acid (NAA) atau 4 amino 3,5,6, trichloropicolinic acid (Picloram) 0.5, 1, 1.5, and 2 mg l^{-1}. Perlakuan kombinasi BA 1 mg l^{-1} dengan NAA 0.5 mg l^{-1} menghasilkan pertambahan tinggi tanaman terbesar. Tanaman menghasilkan jumlah daun terbanyak pada kontrol. Perlakuan BA 1 mg l^{-1} menumbuhkan tunas aksilar terbanyak, sedangkan kombinasinya dengan NAA 1.5 mg l^{-1} merupakan perlakuan yang dapat menghasilkan tunas adventif. Kombinasi BA 1 mg l^{-1} dan NAA 2 mg l^{-1} menginduksi kalus terbaik. Tanaman yang dihasilkan belum membentuk akar, tetapi pertumbuhan lebih lanjut dapat membentuk perakaran dan dapat hidup di luar botol kultur setelah diaklimasi.

Kata kunci: *Nepenthes albomarginata*, tanaman pemakan serangga, kantong semar, kultur pucuk tunas, *in vitro*

INTRODUCTION

Carnivorous plant has an adaptation strategy to unfavorable conditions, mostly to low nutrient availability in wet and acid soils (Adamec 1997), considers having criteria of catching or trapping prey, absorbs metabolites from prey, and utilizes these metabolites for growth and development (Lloyd 1942). The plants consist of over 600 species, out of a total about 300,000 species of vascular plants in the world (Adamec 1997). The genus *Nepenthes* produce pitchers from its leaf tips, consists of 129 species, the largest genus of carnivorous plant in the world (Anonym 2010). There are many new natural and artificial hybrids among the genus *Nepenthes* (D'Amato 1998; Anonym 2010; Merbach & Merbach 2010). The distribution of *Nepenthes* is restricted to tropical areas of the old world. Its habitats are very diverse: limestone cliffs that continually damp, sand areas that have wet and dry season, swamps that under water part of the year, sea shores, epiphytes on tree plants, and creepers on the surface of the soil (Pietropaolo &

Pietropaolo 1986).

These plants have been used for medication, the fluid of unopened pitchers have been used as a laxative, remedy for burns, coughs, inflamed eyes, bed wetter's, fever, stomachache, dysentery, and various skin disorders. The open pitchers have been used as pots to carry water and cook food, the vines have been used as a cordage; and use for ornamental purposes (Perry & Metzger 1980; Pietropaolo & Pietropaolo 1986; Phillips & Lamb 1996; D'Amato1998; Puspitaningtyas & Wawangningrum 2007).

N. albomarginata is one of the carnivorous plants, a tropical plant native to Borneo, Sumatra, and the Malay Peninsular, characterized by coriaceous leaf in texture and lack of petioles, tendrils are up to 20 cm long, an attracted white collar of velvety hairs directly beneath the peristome, pitcher size can be up to 15 cm high, the color usually is green, but there are red, purple, and black forms. It feeds mainly on termites (D'Amato 1998; Moran *et. al.* 2001; Merbach & Merbach 2010).

Nepenthes spp. in Southeast Asia are in danger of extinction because of over exploitation, clear-cut deforesting, area conversion to settlements, mount eruption, climate change, and pollution (D'Amato 1998; Puspitaningtyas & Wawangningrum 2007). These plants could be propagated by seed, stem cutting, air layering, and ground layering (Pietropaolo & Pietropaolo 1986). Sexual propagation by seed has pollination problem because male and female flowers occurred on different plants. Vegetative propagation by cutting or layering takes long time and low results. Vegetative propagation by tissue culture (in vitro) is a good choice to get a lot number of uniform plants in relatively short time in order to conserve, reduce exploitation on wild stocks, and replenish declining populations in the habitat.

Some carnivorous plants had been propagated successfully by tissue culture, such as *Cephalotus follicularis* (Adams *et al*. 1979a), *Dionaea muscipula* (Teng1999; Jang *et al*. 2003), *Drosera intermedia, D. hilaris, D. brevifolia, D. rotundifolia, D. capensis, D. binata, D. omissa, D. peltata, D. indica* (Kulkulczansa 1991; Anthony 1992; Sukamto 1999; Kim & Jang 2004; Jayaram & Prasad 2007), *Drosophyllum lusitanicum* (Goncalves & Romano, 2005), *Nepenthes khasiana* (Latha & Seeni 1994), *Pinguicula moranensis, P. lusitanica* (Adams *et al*. 1979b; Goncaves *et. al*. 2008), and *Urticularia inflexa* (Ram *et al*. 1972). The best formulation of culture medium was ½ MS (Anthony 1992; Kim & Jang 2004; Goncalves *et al*. 2008). There is no report on *N. albomarginata* tissue culture; those successes on other carnivorous plants could be used as references for in vitro propagation of *N. albomarginata.*

MATERIALS AND METHODS

Seeds of *N. albomarginata* originated from West Sumatra, grown in vitro on half strength of macro and micro elements of MS basal medium formulation (Murashige & Skoog 1962). Plantlets grown from the seeds in vitro were cut

their 1 cm shoot tips with five leaves used as explants. Each explant was grown on each culture bottles that contain 20 ml medium of ten series treatments with 12 replications. There were 120 shoot tip explants of *N. albomarginata* for experimental study.

Media culture based on MS basal medium consisted of Mg SO4.7H2O 185 mg l-1, CaCl2.2H2O 220 mg l-1, KNO3 950 mg l-1, NH4.NO3 825 mg l-1, KH2PO4 85 mg l-1, FeSO4.7H2O 13.9 mg l-1, MnSO4.7H2O 11.15 mg l-1, ZnSO4.7H2O 4.3 mg l-1, H3BO3 3.1 mg l-1, glycine 2 mg l-1, vitamin B1 0.1 mg l-1, vitamin B6 0.5 mg l-1, nicotinic acid 0.5 mg l-1, KI 0.415 mg l-1, CoCl2.6H2O 0.0125 mg l-1, CuSO4.5H2O 0.0125 mg l-1, NaMoO4.2H2O 0.0125 mg l-1, myo-inositol 100 mg l-1, sucrose 20 g l-1, and phytagel 2 g l-1 with addition of BA at 1 mg l-1 with or without NAA or Picloram at 0.5, 1, 1.5, 2 mg l-1. The pH was adjusted to 5.7 - 5.8, before it was autoclaved. The medium of 20 ml was poured into 100 ml culture bottle, autoclaved at 1210C and 1 kg cm-2 pressure for ten minutes.

The experimental design was a randomized complete design (RCD) with one factor of hormone doses. There were ten treatments: (1) 0.5 MS (control), (2) BA 1 mg l-1, (3) BA 1 mg l-1 + NAA 0.5 mg l-1, (4) BA 1 mg l-1 + NAA 1 mg l-1, (5) BA 1 mg l-1 + NAA 1.5 mg l-1, (6) BA 1 mg l-1 + NAA 2 mg l-1, (7) BA 1 mg l-1 + Picloram 0.5 mg l-1, (8) BA 1 mg l-1 + Picloram 1 mg l-1, (9) BA 1 mg l-1 + Picloram 1.5 mg l-1, (10) BA 1 mg l-1 + Picloram 2 mg l-1. The cultures were incubated at 26 ± 1 0C under 16:8 photoperiod with TL lamp 40 watt.

The parameter growth of increasing plantlet height, increasing leaf number, axillary shoot number, adventitious shoot number, percentage of dried leaf, percentage of forming calli, quantitative and qualitative calli, and root formation were recorded every 2 weeks until 12 weeks of culture (WOC). Increasing plantlet height was measured by substracting plantlet height to plantlet height 2 weeks before. This measurement was also same for increasing leaf number. Axillary shoot is the shoot grown from node, whereas adventitious shoot is the shoot grown from other parts of the node. Good callus is white color, wet, and can grow further, whereas bad callus is black color, dry, and did not grow further. Data were analyzed with analysis of variance (ANOVA) to know differences among treatments, followed with Duncan's multiple range test (DMRT), Procedure of Statistical Product and Service Solution (SPSS) 12.00 for Windows.

RESULTS

Increasing Plantlet Height and Leaf Number

Hormone treatments were not significantly different on increasing plantlet heights of *N. albomarginata* but the average of plantlet height was higher in control than other treatments on 2 WOC. The explants produced the highest plantlets on 4 WOC in media with addition of BA 1 mg l-1 or its combination with NAA 1 mg l-1. Later on, shoot tips of *N. albomarginata* produced the highest plantlets on 6 - 12 WOC in media with

addition of BA 1 mg l-1 + NAA 0.5 mg l-1. Increasing plantlet height decreased by increasing NAA doses combined with BA; even combination of BA and Picloram at 1 - 2 mg l-1 did not have any increasing plantlets height (Figure 1 A; Table 1).

N. albomarginata shoot tips produced the highest leaf number in control on 2 - 4 WOC. The addition of BA 1 mg l-1 + NAA 0.5 mg l-1 produced the highest leaf number on 6 - 10 WOC. The addition NAA at higher dose than 0.5 mg l-1 inhibited leaf growth (Figure 1 B). Later on, *N. albomarginata* produced the highest leaf number in control on 12 WOC (Table 1).

Axillary and Adventitious Shoot Numbers

Shoot tip explants of *N. albomarginata* have not produced any axillary shoot on 2 WOC. They started to produce axillary shoots at BA 1 mg l-1 treatment or it's combined with NAA 0.5 mg l-1 and NAA 1.5 mg l-1 on 4 WOC. Axillary shoot number at BA 1 mg l-1 was significantly higher compared to those other treatments on 6 - 12 WOC, its combination with NAA inhibited producing axillary buds. The combination of BA and Picloram did not produce any axillary shoot (Figure 2 A; Table 1).

Shoot tip explants of *N. albomarginata* started to produce adventitious shoots in media with addition of BA 1 mg l-1 + NAA 1.5 mg l-1 on 6 WOC (Figure 2 B). The other treatments did not produce any adventitious shoot (Table 1)

Dried leaf

Explants had been cut and grown in media culture, became stress and showed

Table 1. Effects of BA, combined with NAA or Picloram on growth, dried leaf and callus formation of *N. albomarginata* shoot tip on 12 WOC

Hormone Treatments (l^{-1})	Increasing		Shoot Numbers		Dried Leaf (%)	Callus Formation		
	Height (mm)	Leaf Number	Axillary	Adventi.		%	Quantit.	Qualit.
1/2 MS (Control)	3.50 ab	**5.58 a**	0.00 b	0.00 a	24.12 de	0.00 c	-	-
BA 1.0	3.50 ab	4.92 ab	**1.58 a**	0.00 a	9.20 e	0.00 c	-	-
BA 1.0+NA 0.5	**4.08 a**	5.42 a	0.17 b	0.00 a	12.02 e	8.33 bc	+	bad
BA 1.0+NA 1.0	3.58 ab	4.92 ab	0.00 b	0.00 a	39.25 d	8.33 bc	+	bad
BA 1.0+NA 1.5	2.83 b	3.83 bc	0.08 b	**0.25 a**	59.67 c	8.33 bc	+	good
BA 1.0+NA 2.0	1.67 c	3.58 c	0.00 b	0.00 a	61.37 c	**41.67 a**	**++++**	**good**
BA 1.0+Pic 0.5	1.18 c	1.64 d	0.00 b	0.00 a	80.62 b	0.00 c	-	-
BA 1.0+Pic 1.0	0.00 d	0.27 e	0.00 b	0.00 a	94.15 ab	36.36 ab	+++	good
BA 1.0+Pic 1.5	0.00 d	0.00 e	0.00 b	0.00 a	**100.00 a**	25.00 abc	++	bad
BA 1.0+Pic 2.0	0.00 d	0.00 e	0.00 b	0.00 a	**100.00 a**	33.33 ab	++	bad

Notes: Means in the same group followed by the same letter in the columns are not significantly different based on DMRT test at the 5% level

A) B)

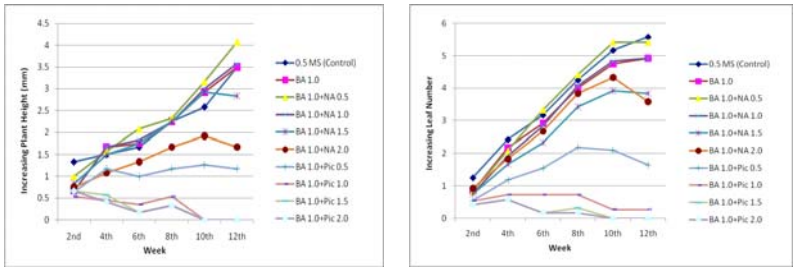

Figure 1. Effect of hormone treatments on increasing plantlet height (A) and increasing leaf number (B) of *N. albomarginata* every 2 WOC

A) B)

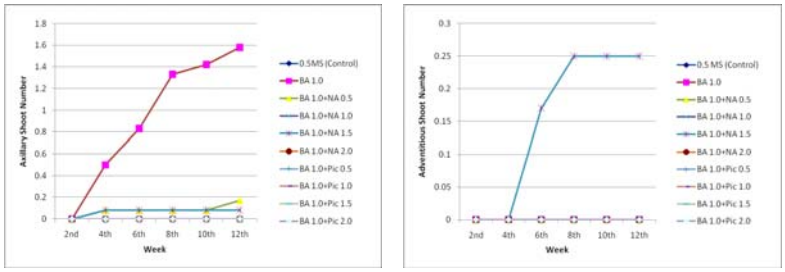

Figure 2. Effect of hormone treatments on axillary shoot number (A) and adventitious shoot number (B) of *N. albomarginata* every 2 WOC

A) B)

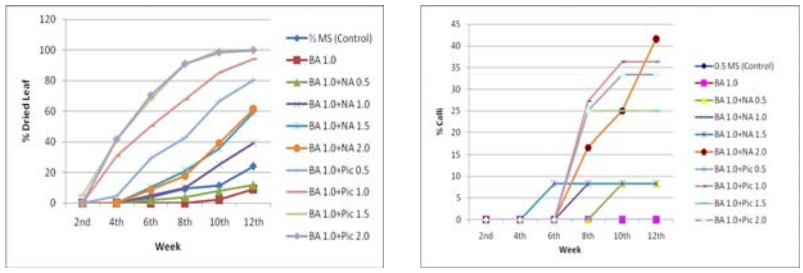

Figure 3. Effect of hormone treatments on dried leaf percentage (A) and calli percentage (B) of N. albomarginata every 2 WOC

drying their leaves 24.12%. The addition of BA 1 mg l-1 and its combination with NAA 0.5 mg l-1 could decrease dried leaf 9.20% and 12.02% consecutively, even though it was not significantly different. Combination of BA 1 mg l-1 with NAA 0.5 - 2 mg l-1 increased substantially the dried leaf, even combination of BA 1 mg l-1 with Picloram 0.5 - 2 mg l-1 caused deadly the explants, especially all explants died at Picloram 1,5 - 2 mg l-1 (Figure 3a; Table 1).

Figure 4. *N. albomarginata* culture in vitro (left) and . *N. albomarginata ac* climatized in vivo (right)

Callus formation

Shoot tip explants of *N. albomarginata* started to produce callus in BA 1 mg l-1 + NAA 1.5 mg l-1 on 6 WOC. Then, producing calli occurred in BA 1 mg l-1 combined with NAA 1 - 2 mg l-1 or Picloram 1 - 2 mg l-1 on 8 WOC (Figure 3b). The best calli production occurred on combination BA 1 mg l-1 with NAA 2 mg l-1 (Table 1). Resulted calli of *N. albomarginata* were wet, white color, and compact on BA 1 mg l-1 + NAA 2 mg l-1, but white color and friable on BA 1 mg l-1 + Picloram 1 mg l-1 treatments.

Root formation

All plantlets derived from the treatments did not produce any root. However, eventually, plantlets grew further could produce pitchers and roots in vitro (Figure 4). These plantlets were acclimatized and could survive in mixture media of soil, sand, compost, coco peat, and burned rice hulls in vivo.

DISCUSSION

The average of plantlet height of *N. albomarginata* was higher in control than other treatments on 2 weeks of culture. It could be the shoot tip explants have not adapted on media with addition of exogenous hormones. Later on, shoot tips of *N. albomarginata* produced the highest plantlets in media with addition of BA 1 mg l-1 + NAA 0.5 mg l-1 on 6 - 12 weeks of culture. It showed the shoot tips have adapted to BA and NAA hormones. Increasing plantlet height decreased by increasing NAA doses combined with BA. It indicated that combination of BA 1 mg l-1 and low dose of NAA (0.5 mg l-1) caused synergism effect. This result was agreed with Windasari (2004) on shoot tip culture of Chrysanthemum morifolium; Goleniowski *et. al*. (2003) on meristem culture of Origanum vulgare x applii. The high dose of NAA greatly stimulate ethylene formation, which it inhibits elongation of stems and roots (Salisbury & Ross, 1978). Combination of BA and Picloram at 1 - 2 mg l-1 did

not have any increasing plantlets height. It because Picloram considers as hormone and also strong herbicide that is stronger than other auxins for inhibiting organogenesis (Davis & Olson 1993) and Picloram disturbs DNA transcription and RNA translation that inhibits producing enzyme for growth regulator (Salisbury & Ross 1978).

N. albomarginata plantlets produced the highest leaf number in control on 2 - 4 weeks of culture, but the addition of BA 1 mg l-1 + NAA 0.5 mg l-1 produced the highest leaf number on 6 - 10 weeks of culture. It showed the plantlets have adapted to BA and NAA hormones and caused synergism effect to improve increasing leaf number on lower dose of NAA. The same result had been reported by Chairunnisa (2004) on increasing leaf number of *Chrysanthemum.* NAA is an auxin that could increase leaf number (Lyndon 1990). However, the addition NAA at dose higher than 0.5 mg l-1 inhibited leaf growth. The similar result also reported by Windasari (2004) on *Chrysanthemum morifolium*. Later on, N. albomarginata produced the highest leaf number in control on 12 weeks of culture. It could be plantlets had been exposure long enough to the hormones that inhibited producing leaf number.

Plantlets of *N. albomarginata* produced axillary shoots that was significantly higher at BA 1 mg l-1 compared to those other treatments on 6 - 12 weeks of culture. It showed that BA is an important cytokinin that releases apical dominance and stimulates outgrowth of axillary shoot (Bidwell 1979). The addition of NAA inhibited activity of BA and also inhibited producing axillary buds. It related to the ratio cytokinin to auxin that is an important to control morphogenesis, high values of ratio cytokinin to auxin stimulate shoot formation (Skoog & Miller 1957). Combination of BA and Picloram did not produce any axillary shoot. It showed that Picloram is stronger auxin than NAA that inhibited axillary shoot formation of *N. albomarginata.*

Plantlets of *N. albomarginata* produced adventitious shoots only in media with addition of BA 1 mg l-1 + NAA 1.5 mg l-1. It showed that combination of BA 1 mg l-1 and NAA 1.5 mg l-1 was the right dose for initiating adventitious shoots. This result has agreed with Bidwell (1979) that interaction of cytokinin with auxin at the right dose could induce organ formation.

The addition of BA 1 mg l-1 and its combination with 0.5 mg l-1 NAA inhibited leaf senescence, because cytokinins including BA could prevent chlorophyll loss (Bidwell 1979) and inhibits leaf senescence (Salisbury & Ross1978; De Klerk 2010). Dried leaf of *N. albomarginata* explants decreased over twice by addition of BA 1 mg l-1. Different result was obtained by Sukamto (2001), which BA mg l-1 increased twice dead explants. It happened because of media, micro-environment (light & temperature) and culture period differences. Combination of BA 1 mg l-1 with NAA or Picloram 0.5 - 2 mg l-1 increased substantially the dried leaf. It happened because auxins (NAA & Picloram) could increase ethylene, high ethylene accumulation in the bottle culture caused leaf senescence (George 1996; De Klerk

2010). NAA and Picloram at high concentration could kill plants and categorize as effective herbicides (Salisbury & Ross 1978).

Shoot tip explants of *N. albomarginata* could produce callus at BA 1 mg l-1 + NAA 1.5 mg l-1, BA 1 mg l-1 + NAA 1 - 2 mg l-1 or Picloram 1 - 2 mg l-1 but did not produced any callus on control, BA 1 mg l-1, BA + NAA or Picloram at doses 0.5 mg l-1. It showed shoot tip tissues of *N. albomarginata* did not have enough auxin endogen. Ratio cytokinin to auxin is an important to control morphogenesis; low values of ratio cytokinin to auxin stimulate callus formation (Skoog & Miller 1957). Plant tissues formed callus because of wounded tissue or stress (Kyte 1990). Resulted calli of *N. albomarginata* from BA 1 mg l-1 + NAA 2 mg l-1 were wet, white color, and compact, whereas from BA 1 mg l-1 + Picloram 1 mg l-1 were white color and friable. Calli structures are important for further morphogenesis. Compact calli structures are usually produce adventitious shoot, whereas friable structure indicated embryogenic calli (Torres 1989). The similar results of Picloram effect were reported by Vaverde *et al*. (1987) in embryogenesis of pejibaje palm; Omar & Novak (1990) in callogenesis of date palm; Davis & Olson (1993) in organogenesis of spurge.

All plantlets derived from the treatments did not produce any root. This experiment was only use 0.5 MS and BA + NAA or Picloram but did not use NAA alone or other auxins. Adventitious root development is usually promoted by auxin treatment (Salisbury & Ross 1978). It could be affected by inhibition of BA cytokinin to auxin's function or MS strength, and the auxin type. This result confirmed by Goncalves & Romano (2005) that the highest rooting frequency was 0.25 MS and indole-3-butyric acid (IBA) at 0.2 mg l-1 on *Drosophyllum lusitanicum* culture; Goncalves *et al*. (2008) reported that 0.25 MS and indole acetic acid (IAA) at 0.2 mg l-1 produced the highest rooting frequency, NAA at 0.5 mg l-1 suppressed completely rooting response on Pinguicula lusitanica culture.

This experiment revealed that shoot tip culture of *N. albomarginata* could grow on 0.5 MS media with or without addition of hormones, but axillary and adventitious shoots could not formed on control. The highest increasing height of plantlets occurred on combination BA 1.0 mg l-1 and NAA 0.5 mg l-1, whereas the highest increasing leaf number on control. The best formation of axillary shoots occurred on BA 1.0 mg l-1, whereas adventitious shoot only occurred on BA 1 mg l-1 + NAA 1.5 mg l-1. The best callus occurred on BA 1 mg l-1 + NAA 2 mg l-1. All plantlets did not produce root but eventually, plantlets grew further could produce pitchers and roots in vitro, also survived after acclimatization in vivo. This successful propagation of *N. albomarginata* could be used for conservation and increasing population.

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