

IN-VITRO CULTURE FOR REGENERATION OF *Melia azedarach* L., USING AGITATED LIQUID MEDIUM

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ABSTRACT

This study investigated the applicability of liquid medium in direct organogenesis of in-vitro culture of *Melia azedarach* L. Explants were collected from in-vitro multiplication originating from aseptically germinated seedling and from a 48 - years old mature tree. For adventitious shoot differentiation, 2 mm length of excised explants were cultured on liquid medium of MS (*Murashige and Skoog's*) basal medium supplemented with 28 combinations of hormone BAP (*6-benzylaminopurine*) and NAA (*α -naphthaleneacetic acid*). Differentiated shoots were subsequently cultured for shoot elongation in solid medium using MS basal medium supplemented with hormone BAP individually as well as in combinations with NAA. Finally, rooting culture were done using MS medium supplemented with IBA (*3 indolebutyric acid*). The results showed that the rate of shoot organogenesis of *M. azedarach* could be obtained through agitated liquid medium culture technique. The combination of hormone BAP 0.1-1 μ M and NAA 0.01-0.1 μ M are induced more adventitious shoot at a rate of 5 shoots / 2 mm length size of explants are transferring into rooting medium containing IBA 4.92 μ M.

Key words: *Melia azedarach* L., liquid medium, in-vitro culture, direct organogenesis, adventitious shoots.

I. INTRODUCTION

Melia azedarach L., (Persian Lilac, Ghora Neem) is one of the important species in Meliaceae family which primary native to the Orient, especially in Iran, China, India, Japan, and naturalized in many sub-tropical regions of the world (Kelvey, 1928). *M. azedarach* is a high priority multi-purposes tree due to its capability to produce valuable timber that is resistance to termites, fodder, green manure, and oil from seed. This species also contains therapeutic, insecticidal properties and some several compounds of limonoids (Itokawa *et al.*, 1995; Huang *et al.*, 1996). Traditionally, *M. azedarach* is propagated using seeds, however, the rate of multiplication is low due to the recalcitrant nature of the seeds. Moreover, the species bears seed only during summer (Thakur *et al.*, 1998; Deb, 2001).

Rapidity and high rate of multiplication from small size explants will be a great breakthrough to propagate improved material in biotechnology and tree improvement program of *M. azedarach*. Considering the limitation of available meristem tissue collected from mature trees for clonal propagation. For that reason, selecting tissue culture technique using effective medium culture will be necessary. This technique may have some advantages that could eliminate several disadvantages in the uses of solid medium, such as facilitate

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gaseous exchange in medium, remove polarization of tissue due to gravity, eliminate nutrient gradient in medium and surface of explants (Yeoman, 1973). Another advantage is the benefit of effective explants size with the opportunity to culture relatively small size explants to produce more new buds primordial. At present, there is no report on tissue culture using agitated liquid medium for *M. azedarach* propagation.

This study was carried out to develop a direct organogenesis of in-vitro culture technique for *M. azedarach*, using agitated liquid medium. The study was focused to observe the uses of agitated liquid medium for the initiation of new buds formation of *M. azedarach* using stem explants excised from aseptically germinated seedling. Simple comparison was also made with stem explants originated from mature tree.

II. MATERIAL AND METHODS

A. Explants

Explants were collected from in-vitro multiplication of aseptically germinated seedling as well as mature tree. Seeds were surface-sterilized by immersing in 70 % sodium hypochloride for 20 minutes, followed by washing 5 times with sterile distilled water. Sterilized seeds were germinated aseptically into 25 x 150 mm tubes containing half strength MS (*Murashige and Skoog's*) basal medium supplemented with 2 % sucrose and solidified with 2.4 g/l gelrite. The axillary shoots from the same individual seedling were then excised and cultured on MS basal medium supplemented with BAP (*6-benzylaminopurine*) 1 μM , 2 % sucrose and solidified with 2.4 g/l gelrite for shoot elongation and for further multiplication. Stems (with no axillary bud) from elongated shoot plantlets were excised and cut into small segments in 2 mm length as explants. In the case of mature tree, stem explants were prepared using multiplication of explants collected from sprouted shoot of 48 years age tree branches.

B. Media Culture Preparation and Culture Conditions

All media were adjusted to pH 5.8 and sterilized using autoclave at 121°C for 20 minutes. All cultures were maintained under white fluorescent lights at an irradiance of 50 $\mu\text{molm}^{-2}\text{s}^{-1}$ PPFD (photosynthetic photon flux density), with a 16 hours photoperiod and temperature 25°C.

1. Adventitious shoot differentiation

Study was carried out in two separate researchs (hereinafter referred as Research 1 and Research 2). All 2 mm length excised explants were cultured on liquid medium of MS basal medium containing 2 % sucrose (without agar) supplemented with hormone. There were 16 combinations of hormone used in Research 1: combination of BAP 1, 3, 6, 10 μM and NAA (*α -naphthaleneacetic acid*) 0, 0.01, 0.1, 1 μM . In Research 2, 12 combinations of hormone were used: combination of BAP 0.1, 0.5, 1, 5 μM and NAA 0, 0.01, 0.1 μM . There were 10 samples of explants in three replications for each combination of hormone treatment. All cultures were horizontally agitated using shaker in 125 rpm, with continuous immersion. After 5 weeks of culturing, the adventitious shoot differentiation were observed.

2. Shoot elongation

The differentiated adventitious shoot explants in agitated liquid medium were transferred into solid medium of shoot elongation (solidified with 2.4 g/l gelrite) containing 2% sucrose with the same basal medium as for adventitious shoot differentiation. The medium were supplemented with hormone BAP 0.01, 0.1, 1 μM individually, and BAP 1 μM in combination with NAA 0.01 μM . Sub-culture for multiplication of elongated shoot explants were done twice using the same media, with an interval of 2 weeks for each culture.

3. Rooting

The elongated shoots of 2 - 2.5 cm length were excised, and transferred into vermiculated rooting medium containing half-strength MS medium supplemented with IBA (3 *indolebutyric acid*) 4.92 μM (Thakur *et al.*, 1998), 2 % sucrose (without agar) for 4 weeks culturing.

III. RESULTS AND DISCUSSION

A. Adventitious Shoot Differentiation

Adventitious shoot of *M. azedarach* were well differentiated through in-vitro culture using agitated liquid medium. Explants cultured in low concentration of BAP (1 μM in Research 1), and (0.1 - 1 μM in Research 2), all of which in combination with NAA 0.01 - 0.1 μM , produced more adventitious shoot (ranging from rates of 80 % to 100 %) than those in high concentrations of BAP (Figure 1). In Research 1, although between the low concentration of BAP (1 μM) in combination with NAA 0.01 μM and that in combination with NAA 0.1 μM was significantly different, both combinations showed high response for adventitious shoot differentiation (the top of two). In the case of Research 2, there was no significant difference in response for shoot differentiation between the low concentrations BAP (<1 μM), either in combination with NAA 0.01 μM or in combination with NAA 0.1 μM . Similar as to previous study using solid medium (Nirsatmanto, 2002), this result indicated that the effects of increasing BAP concentration tends to reduce the response for shoot initiation. However, with no supplementation of NAA (0 μM), or in supplementation of high concentrations NAA (1 μM), the effectiveness of low concentrations BAP was also much reduced for shoot initiation.

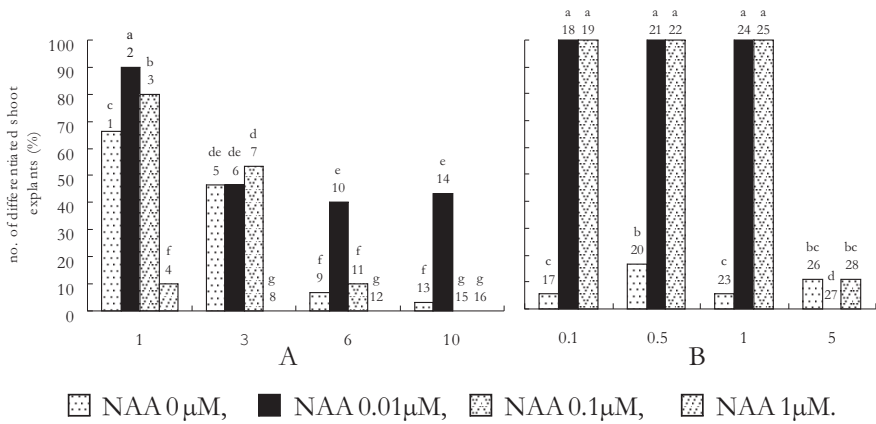


Figure 1. The effects of 28 combinations of hormone BAP and NAA on differentiated adventitious shoot using agitated liquid medium.

Remarks : Research 1 (A) and Research 2 (B). Data were collected after 4 weeks in culture, with the total 30 explants/ treatment. Number's above Bar is the number of combinations of hormone. Bar having different letter (a, b,...) in each (A) and (B) indicate significant differences at $p < 0.05$.

Adventitious shoots differentiation was well developed without any appearance of induced callus. The differentiations were started after 3 weeks in culture, and it was visually observable after 5 weeks in culture with more than 5 shoots per explants (Figure 2). In the same scale of explants length, the rate of differentiated adventitious shoot obtained from this agitated liquid medium technique was higher than that from solid medium resulted in previous study (Nirsatmanto, 2002). In previous study, the 1 cm explants length produced around 7 - 8 shoots/explants. Whereas in this study, with 2 mm explants length produced more than 5 shoots/explants or in equivalent to more than 25 shoots per 1 cm length explants segment. This indicated that agitated liquid medium technique is more effective and applicable for direct shoot organogenesis of *M. azedarach*.

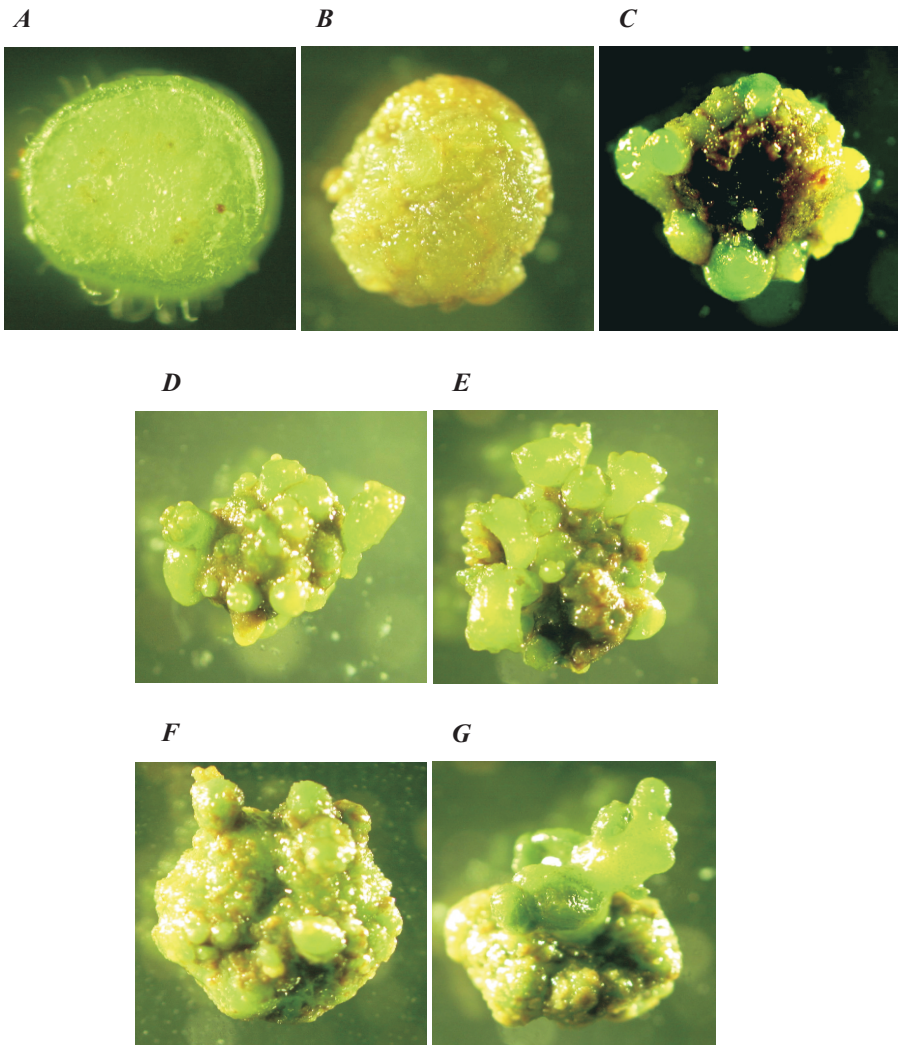


Figure 2. The initial morphology and development of differentiated adventitious shoot of *M. azedarach* cultured on agitated liquid medium containing combinations of hormone BAP $1\ \mu\text{M}$ and NAA $0.01\ \mu\text{M}$.

Remarks: A, B, C, D, and E are the morphology from juvenile explants cultured in 0, 2, 3, 4, and 5 weeks, respectively. F and G are the morphology from mature tree explants cultured in 3 and 4 months, respectively.

In regards to the explants maturity, agitated liquid medium could be used also to propagate *M. azedarach* using explants collected from 48 age years of mature tree. However, the regeneration rate was notably low (data not shown) as compared to juvenile explants. Moreover, it needs a longer time, about 3-4 month in culture, to be visually observable (Figure 2: F and G).

B. Shoot Elongation and Rooting

Differentiated adventitious shoot explants, cultured in medium no. 1, 2, 3, 5, 6, 7 and 14 (Figure 1), were transferred into solid medium of shoot elongation. All differentiated shoot explants previously cultured on medium no. 1, 2 and 6 showed high survival rate on all combinations of hormone during shoot elongation (Table 1). The longest shoot was observed at about 2.5 cm length in medium containing BAP 1 μ M in combination with NAA 0.01 μ M after twice sub-culture on the same medium (Table 1 and Figure 3). This combination of hormone appeared to be more effective than others due to the high response not only for shoot elongation, but also for initiating adventitious shoot in agitated liquid medium (Figure 1).

Table 1. The effects of combinations of hormone BAP and NAA

Hormone μ M		Shoot elongation medium number s, Survival rates (%) and shoot length (cm)						
BAP	NAA	1	2	3	5	6	7	14
0.01	0	100	100	0	66	100	50	66
		(1.0)	(0.5)	(-)	(0.3)	(2.0)	(0.3)	(0.3)
0.1	0	100	100	20	100	100	100	100
		(1.5)	(0.5)	(0.5)	(1.5)	(1.5)	(1.0)	(0.3)
1	0	100	100	40	100	100	100	100
		(1.5)	(1.5)	(0.5)	(1.0)	(0.5)	(2.0)	(1.0)
1	0.01	100	100	20	100	100	100	100
		(2.5)	(2.5)	(0.5)	(0.5)	(0.5)	(0.5)	(0.3)

Remarks: Survival rates above and shoot elongaton in parentheses.
Data were collected from the second sub-culture of shoot elongation with culture interval 2 weeks.

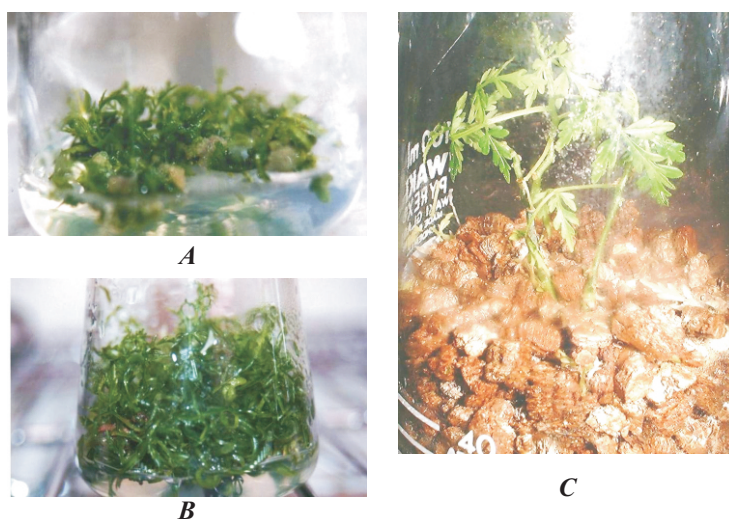


Figure 3. The growth of differentiated shoots of *M. azedarach* after 2 weeks and 4 weeks.
Remarks : A = Adventitious shoot elongation after 2 weeks
B = Adventitious shoot elongation after 4 weeks
C = The excised shoots rooted on vermiculate containing rooting medium

The excised shoots produced roots after transferring into half strength MS supplemented with IBA $4.92 \mu\text{M}$ for 4 weeks (Figure 3: C). In the case of elongated shoot from mature explants, rooting was not observed yet after culturing in 4 weeks. Bonga (1982) and Hacket (1987) reported the difficulties of propagation using material collected from mature woody plant due to inhibition of material sterilization, shoot and rooting differentiation.

IV. CONCLUSION

1. This study showed that the rate of shoot organogenesis of *M. azedarach* could be achieved using agitated liquid medium culture technique.
2. The combination of hormone BAP $0.1-1 \mu\text{M}$ and NAA $0.01 - 0.1 \mu\text{M}$ revealed to be effective to induce more adventitious shoot directly from 2 mm length of small size explants.
3. The differentiated shoots should be transferred into other solid medium supplemented with the combination of hormone BAP $1 \mu\text{M}$ and NAA $0.01 \mu\text{M}$ as in adventitious shoot differentiation medium, for shoot elongation. Subsequently, the elongated shoot could produce roots after transferring into rooting medium containing IBA $4.92 \mu\text{M}$.

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