GENETIC TRANSFORMATION OF *Melia azedarach* L., USING *Agrobacterium* MEDIATED TRANSFORMATION

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ABSTRACT

This study was subjected to observe the possibility of introducing specific foreign genes into *Melia azedarach* L., using *Agrobacterium* mediated transformation. *Agrobacterium tumefaciens* used in this study consisted of strains of EHA105 (vector plasmid pBIsGFP) and EHA105 (vector plasmid pBsGFP) to observe the possibility of introducing genes, and strains of EHA101 (vector plasmid pIG121-Hm) and LBA4404/ferritin (vector plasmid pBG-1) to observe the shoot organogenesis after genes transformation. Explants were collected from one cm in length excised stem of *in-vitro* plantlets. The results of the study showed that genetic transformations of *M. azedarach* could be potentially developed using *Agrobacterium tumefaciens* strains : EHA105 (pBIsGFP or pBsGFP), EHA101 (pGI121-Hm) and LBA4404/ferritin (pBG-1). The expression of GFP (*green fluorescence protein*) signal worked successfully in this transformation with 40% of transformation rate for pBIsGFP and 20 % for pBsGFP. The application of *Agrobacterium* strains of EHA101 (pIG121-Hm) and LBA4404/ferritin (pBG-1) which contained specific gene of kanamycin resistance and iron accumulation for plant growth improvement showed that adventitious shoot was well induced and elongated on the rate of 30 - 60 % of explants after genes transformation.

Keywords: *Melia azedarach* L., *in-vitro* culture, genetic transformation, *Agrobacterium tumefaciens*

I. INTRODUCTION

Genetic engineering through genetic transformation has played important role for breeding purposes in advance tree improvement program. It is due to the success of genetic transformation which may introduce such novel genetic character resistance to disease, insect, and herbicide. This becomes a very important part of producing new improved variety in forest tree species. The success of genetic transformation requires a high regenerative system of organogenesis, because of low efficiency of transformation due to kind of methods that was applied to introduce foreign gene into plants, such as using *Agrobacterium*, particle bombardment or electroporation (Kawazu *et al.*, 1996).

Introducing specific foreign genes into plant through genetic engineering could increase the growth of transformed plant due to increasing the content of iron inside plant metabolism (Goto *et al.*, 2000). Genetic transformation technology has proved the benefit of transformed plant for increasing productivity. However, the considerable progress of genetic transformation study for forest tree is still quite limited which are restricted to poplar and eucalypts (Cheliak and Rogers, 1990; Harcourt *et al.*, 2000). To date, study on genetic transformation for *Melia azedarach* L., has not yet been reported.

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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 region of the world (Kelvey, 1928). *M. azedarach* is high priority multi-purposes tree due to capability to produce valuable timber resistance to termites, fodder, green manure, and oil from seed. This species also contains therauptic, insecticidal properties and some several compounds of limonoids (Itokawa *et al*., 1995; Huang *et al*., 1996).

In term of economic benefits of *M. azedarach* L., improving quality by applying the genetic transformation technology is necessary. It is supported with the success of shoot organogenesis of this species which are a prerequisite matter for the success of genetic transformation (Nirsatmanto, 2002). However, to date, study on genetic transformation for *M. azedarach* L has not yet been reported. In the present study, we observed the possibility of introducing specific foreign genes into *M. azedarach* using *Agrobacterium* mediated transformation. This method was selected due to it has become widely used for genetic transformation, and it also showed higher gene transfer efficiency than using non-bacterium methods (Muramatsu *et al*., 1998).

II. MATERIAL AND METHODS

A. Explants

Explants used in this study were collected from *in-vitro* plantlets of *M.azedarach*. Stems (with no axillary bud) were excised from the plantlets and cut into about one cm in length as explants.

B. Agrobacterium strains

In the first stage, the GFP (*Green Fluorescence Protein*) of *A. tumefaciens* strains of EHA105 (vector plasmid pBIsGFP) and EHA105 (vector plasmid pBsGFP) were used to observe possibility of introducing genes into *M. azedarach*. Thereafter, the *A. tumefaciens* strains of EHA101 (vector plasmid pIG121-Hm) and LBA4404/ferritin (vector plasmid pBG-1) were applied to observe the shoot organogenesis after transformation (Otani *et al*., 1998; Goto *et al*., 1998). Before co-cultivation of the bacteria with the explants, *A. tumefaciens* strains of EHA101 and LBA4404 were grown overnight at 25°C in LB (Luria Bertani medium)-antibiotic agar medium containing 100 mg/l kanamycin and 20 mg/l of rifampicin. In the case of two *A. tumefaciens* strains of EHA105, they were grown in absence of rifampicin.

The signal of pBIsGFP will only detect the inserted genes into plant without performing signal for any other organs, while the signal of pBsGFP will detect not only the inserted genes, but also agrobacterium that might be still remained in explants after co-cultivation. The EHA101 and LBA4404/ferritin are the genes with a function to increase the kanamycin resistance and the accumulation of iron content in plant for increasing the growth. (Hood *et al*., 1986; Goto *et al*., 1998)

C. Transformation procedure

Explants were immersed with agitation for 2 minutes in MS (*Murashige and Skoog's*) medium containing overnight grown bacteria that was grown in overnight, supplemented with 2% sucrose, BAP (*6-benzylaminopurine*) 1 μM, NAA (*α-naphthaleneacetic acid*) 0.01 μM (Nirsatmanto, 2002), and 10 mg/l of acetosyringone. The immersed explants were then blotted drying using sterile filter papers and transferred into solid MS medium supplemented with the same concentration of hormone, sucrose, and acetosyringone for co-cultivation. The co-cultivation culture was maintained under 28°C with 0 light intensity (dark).

After three days of co-cultivation, explants were rinsed with agitation for three times (2 minutes interval) using 500 mg/l of carbenicilin. After blotted drying using sterile filter papers, explants were transferred to selection medium containing MS medium supplemented with BAP 1 μM, NAA 0.01 μM, sucrose 2%, kanamycin 100 mg/l, rifampicin 20 mg/l and carbenicilin 500 mg/l for agrobacterium strains of EHA101 and LBA4404, and without riffanpicin for the two of EHA105. Investigation was done every week to observe the differentiation of transformed shoots. In the case of strain of EHA105 (pBIsGFP and pBsGFP), observation was done using the fluorescence photographs microscope. Gene transformations for strains of EHA101 (pIG121-Hm) and LBA4404 / ferritin (pBG-1) were done twice (hereinafter referred as Research 1 and Research 2).

III. RESULTS AND DISCUSSION

A. Strains of EHA105 (vector plasmid pBIsGFP) and EHA105 (vector plasmid pBsGFP)

Observation using Fluorescence Photographs of Nikon's P-FLA Fluorescence Attachment with the stereoscopic microscope SMZ800 showed that the expression of EHA105 (pBIsGFP and pBsGFP) occurred in the segment of stem explants of *M. azedarach* after two weeks of culturing into selection medium. The bright fluorescent signal which indicated the inserted of gene transformation into explants was shown in Figure 1.

The expression of pBIsGFP into stem explants indicated that transformation rate was higher than that of pBsGFP: 40 % for pBIsGFP and 20 % for pBsGFP (Table 1). It means that *A. tumefaciens* strain EHA105 with vector plasmid pBIsGFP seemed to be more effective for mediated transformation of *M. azedarach* than that EHA105 with vector plasmid pBsGFP. Moreover, the expression of pBsGFP itself is probably showed the signal of *agrobacterium* which might be still remained on surrounding explants after co-cultivation.

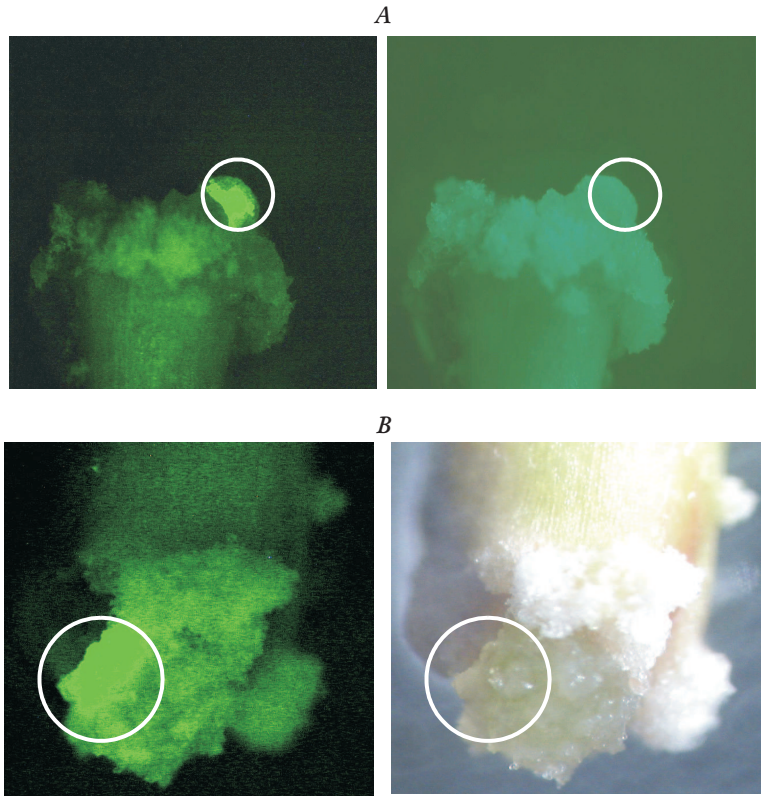

Figure 1. The expression of EHA105 (pBIsGFP and pBsGFP) into stem explants of *M. azedarach* after two weeks culturing on selection medium: ***(A)*** the inserted gene transformed using strain of EHA105 (pBIsGFP), ***(B)*** the inserted gene transformed using strain of EHA105 (pBsGFP). Remarks : The clearly green colours (*circle*) is bright fluorescent signal indicating the inserted of gene transformation. The right hand side pictures are the whole explants with detail position of inserted gene.

Table 1. The effects of *A. tumefaciens* strains of EHA105 (pBIsGFP and pBsGFP) on transformation rate of *M. azedarach* stem explants after culturing into selection medium containing BAP 1 μM, NAA 0.01 μM, kanamycin 100 mg/l, and carbenicilin 500 mg/l.

Agrobacterium strain	Number of explants	Number of explants showing the expression of GFP after transformation	Frequency of transformation (%)
EHA105 (pBIsGFP)	10	4	40
EHA105 (pBsGFP)	10	2	20

The expression of GFP signal in this study indicated that foreign gene has been potentially introduced into *M. azedarach* explants. However, this study is the first application of GFP transformation system for *M. azedarach*. Therefore further study is still necessary to improve the efficiency of GFP system for developing *M. azedarach* genetic transformation.

B. Strains of EHA101 (vector plasmid pIG121-Hm) and LBA4404/ferritin (vector plasmid pBG-1)

The growth of adventitious shoot of *M. azedarach* after transformation using *A. tumefaciens* strains of EHA101 and LBA4404 showed that the first differentiated adventitious shoots were observed after three weeks culturing in selection medium containing 100 mg/l of kanamycin, 20 mg/l of rifampicin and 500 mg/l of carbenicilin. Within 10 weeks (Research 1) and 6 weeks (Research 2) of culture transformation, the differentiated adventitious shoot rates decreased with the time of culture for *A. tumefaciens* strain EHA101: 65 % in 6 weeks and 45.5 % in 10 weeks. On the other hand, the rates of strain Lb4404 slightly increased with the time: 35.7 % in 6 weeks and 44.4 % in 10 weeks (Table.2). Goto *et al.* (2000) reported that higher concentration of kanamycin (100 mg/l) and longer growth period was required for increasing selection transformation using LBA4404 / ferritin.

Table 2. The effects of *A. tumefaciens* strains of EHA101 (pIG121-Hm) and LBA4404 (pBG-1) on transformation rate of *M. azedarach* stem explants after culturing into selection medium containing BAP 1 μM, NAA 0.01 μM, kanamycin 100 mg/l, rifampicin 20 mg/l and carbenicilin 500 mg/l.

Agrobacterium strain	Number of explants	Number of differentiated shoots explants after transformation	Frequency of transformation (%)
Research 1			
EHA101 (pIG121-Hm)	22	10	45.5
LB4404 (pBG -1)	18	8	44.4
Research 2			
EHA101 (pIG121-Hm)	40	26	65
LB4404 (pBG -1)	28	10	35.7

Note: The data on Research 1 were collected after 10 weeks, while the data on Research 2 were collected after 6 weeks culturing on selection medium.

The observation on differentiated adventitious shoots grown in selection medium showed that the rates of shoots differentiation were slow (Figure 2). Shoot growth inhibition in this study might be due to the effects of the antibiotic of kanamycin, rifampicin and carbenicilin that were used during transformation.

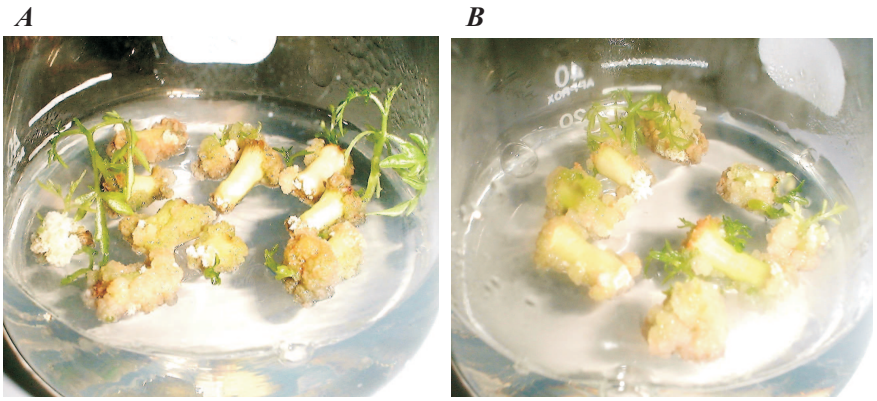

Figure 2. The growth of differentiated adventitious shoot of *M. azedarach* for kanamycin resistance after transforming using *A. tumefaciens*: ***(A)*** transformed using EHA101 (pG121-Hm), ***(B)*** transformed using strain of LBA4404 (pBG-1). Remarks : The first transformed shoots were differentiated after three weeks culture on selection medium containing BAP 1 μM, NAA 0.01 μM, kanamycin 100 mg/l, riffanpicin 20 mg/l, and carbenicilin 500 mg/l. Photos were taken on six weeks culture.

The observation was done in the initial stage of transformed shoots differentiation. Therefore to confirm whether the gene was clearly transformed into new plantlets, the transformed adventitious shoots should be analyzed later by excising and re-culturing into the same selection medium and verified using a technique of DNA molecular, such as PCR or Southern blotted analysis.

IV. CONCLUSIONS

1. The results of the study showed that the genetic transformations of *M. azedarach* could be potentially developed using mediated transformation of *A. tumefaciens* strains : EHA105 (vector plasmid pBIsGFP or pBsGFP), EHA101 (vector plasmid pGI121-Hm) and LBA4404/ferritin (vector plasmid pBG-1), with selection marker resistance to kanamycin.
2. Detection of the possibility for introducing foreign gene into *M. azedarach* explants using GFP *agrobacterium* strains of EHA105 binary vector pBIsGFP and pBsGFP showed that the expression GFP signal worked successfully with transformation rate of 40 % for pBIsGFP and 20 % for pBsGFP. It indicated that foreign gene could be potentially introduced into *M. azedarach* explants.
3. The application of *agrobacterium* strains of EHA101 (pIG121-Hm) and LBA4404/ferritin (pBG-1) mediated transformation which contains specific gene for plant growth improvement (kanamycin resistance and iron accumulation) showed that adventitious shoot was well induced and elongated on 30 – 60 % of *M. azedarach* explants after transformation.

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