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Full Length Research Paper

Transient expression of β-glucuronidase reporter gene in *Agrobacterium*-inoculated shoots of various teak clones

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Agrobacterium tumefaciens **strain LBA4404 carrying the pBI.121 binary plasmid was used in transformation to introduce the *gus* (ß-glucuronidase/GUS) gene into teak shoot-tissues. *In vitro* regenerated shoots from various teak clones, i.e. the ITB, GT, P97, P96, P75, P20, and P108 clones were vacuum-infiltrated for 5 min in the suspension culture of *A. tumefaciens*. Seven days after selection period, the evidence of GUS activity in inoculated teak shoot-tissues was histochemically confirmed. The percentage of GUS-positive shoots per total numbers of evaluated shoots was calculated to estimate the efficiency of transient-transformation. Results indicated that the percentage of GUS-positive shoots varied among teak clones. The highest percentage of shoots with GUS activity (94.6%) was observed from the P108 clone and the lowest percentage (38.9%) was from the P20 clone. High frequencies of GUS expression were observed mostly in leaf and internodal tissues of teak shoots and less detected in young tissues of shoot-tips.**

Key words: ***Agrobacterium***-mediated transformation, ***Tectona grandis*** L.f., teak shoot-cultures.

INTRODUCTION

Teak (***Tectona grandis*** L.f.) is an important tree species for forest plantations in the tropics and famous worldwide because of its high value wood. Genetic improvement of teak by conventional breeding is still an obstacle due to the long reproductive cycle. It takes more than a decade from the initiation of a genetic improvement program until improved seeds are available and needs another 4-5 decades until the timber from the first rotation of improved planting stock is harvested (Suseno and Wibisono, 2000). Therefore, special attention must be given to the use of genetic manipulation for producing genetically engineered teak to pass the long period required for natural genetic crosses and selection.

Successful application of genetic transformation procedures in forest tree species has been reported and mostly achieved using ***Agrobacterium tumefaciens*** as a mediator. Procedures for ***Agrobacterium***-mediated transformation have been developed for sandalwood (Lakhsmi-Sita et al., 1998), ***Populus*** spp. (Dai et al., 2003; Thakur et al., 2005) and pine (Cerda et al., 2002; Charity et al., 2002; Tang et al., 2004). However, no report of successful genetic transformation of teak has been published to date.

In attempts to develop the transformation procedure of teak, we studied the regeneration ability of teak callus tissues to develop shoots and found that the frequency of adventitious shoot formation from teak callus was very low (Widiyanto et al., 2003; Widiyanto et al., 2005). As a matter of fact, ***in vitro*** propagation of teak has been successfully developed using pre-existing shoots and single-node cuttings (Tiwari et al., 2002). Therefore, to overcome the technical difficulty in regenerating transgenic clones, we consider the use of ***in vitro*** cultured of teak shoots as the target explants in transformation that mediated by ***A. tumefaciens***.

In ***Agrobacterium*** mediated transformation, the suc-

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Abbreviations: GUS, β-glucuronidase; **PT,** Putative transformed-shoots; **TE,** Transient-transformation efficiency.

cessful bacterial plant host interaction becomes the most critical factor. The compatible interaction between ***Agrobacterium*** and plant cells has been known to be influenced by many factors including specific plant host factors. Many studies showed that the genotypic variation of plant clones, the difference responses of host-plant tissues to specific bacterial strain, and the compatibility between specific bacterial strains with specific plant organs or tissues should be taken into consideration in ***Agrobacterium***-mediated transformation (Boase et al., 2002; Yu et al., 2002; Zaldívar-Cruz et al., 2003).

The objective of our work reported here was to study the effects of genotypic variation of clones and specificity of organ-tissues of shoots on the efficiency of ***Agrobacterium***-mediated transient transformation of ***in vitro*** cultured teak shoots. In this study, the transient expression of ***gus*** (β-glucuronidase/GUS) gene in teak tissues was used to estimate the transient transformation efficiency (TE) of ***Agrobacterium***-inoculated teak shoots.

MATERIALS AND METHODS

Plant material and culture media

Two week-old in vitro cultured teak shoots consisting of 3 - 5 young tender expanding leaves (Figure 1) regenerated from various teak clones, i.e. the ITB, GT, P97, P96, P75, P20, and P108 clones, were used as the target explants in transformation and subjected to be inoculated with A. tumefaciens. Shoot cultures of the ITB clone were isolated and in vitro regenerated from a 46 year-old teak tree grown in our university campus area. The GT shoot cultures were originally isolated from the 'Golden Teak' (GT) trees grown in the field-area of SEAMEO-BIOTROP, Bogor, Indonesia. The P97, P96, P75, P20, and P108 teak shoot cultures were isolated from selected elite teak clones (Plus Teak clones) obtained from the Perum Perhutani Teak Plantation, Indonesia. All teak shoot cultures were micro-propagated through the single-node micro-cutting technique following the method as described by Tiwari et al. (2002).

To maintain teak shoot cultures, shoot induction medium (SIM) containing inorganic salts and vitamins based on Murashige and Skoog (1962) supplemented with 22.2 µM N^6-benzyladenine and 3% sucrose was used in experiments. Selective culture (SC) media, composed of SIM containing 200 mg/l carbenicillin and 50 mg/l kanamycin, were used to eliminate bacteria after inoculation and select transgenic clones resistant to kanamycin. Carbenicillin and kanamycin were filter-sterilized and added to the medium after autoclaving and cooling to 40-50^{o}C. Culture media were adjusted to pH 5.8 with drops of 1 N NaOH or 1 N HCl and added with 0.25% Gelrite® (Sigma Co.) to solidify. All culture media were autoclaved at 121^{o}C under a pressure of 1.2 kg/cm^2 for 15 min. Teak shoot cultures were maintained in a culture room at 25 ± 2ºC, under a continuous light condition emitted by 40W Philips cool white fluorescent tubes.

Bacterial cultures

A. tumefaciens strain LBA4404 was used in experiments and served as the hosts for the pBI.121 binary plasmid (Clontech Laboratories Inc.). The binary plasmid contains the ***nptII*** (neomycin phosphotransferase II) and ***gus*** (β-glucuronidase/GUS) genes within its T-DNA borders. The ***nptII*** gene that provides resistance to kanamycin was driven by the nopaline synthase (NOS) promoter and terminator sequences. The ***gus*** gene was driven by the cauliflower mosaic virus (CaMV) 35S promoter and NOS terminator sequences (Jefferson et al., 1987). In this research, the expression of ***gus*** gene was used as a visual marker in transformation. Bacteria were suspended in YEB (Yeast Extract Broth) medium supplemented with 100 mg/l streptomycin, 100 mg/l rifampicin and 50 mg/l kanamycin for selection of the pBI.121 plasmid. ***Agrobacterium*** filtrates were cultured at 28^{o}C over night (Lakhsmi-Sita et al., 1998). Before being used in transformation, the bacterial suspension was centrifuged and the bacterium-cell pellets were re-suspended in fresh YEB liquid medium without antibiotics. The culture of ***A. tumefaciens*** LBA4404 without binary plasmid was used as the control treatment.

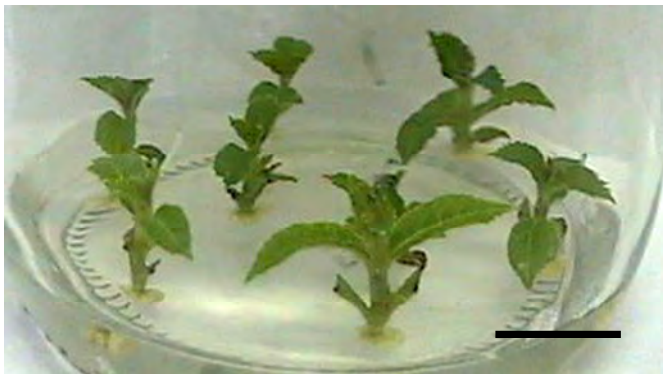

Figure 1. Two week-old teak shoots consisting of 3 - 5 young tender leaves cultured on shoot induction medium. Bar = 10 mm.

Inoculation and infiltration

The basic procedure of bacterial infiltration was using the vacuum-infiltration technique following the procedure described by Charity et al. (2002). Before the vacuum-infiltration was applied, the sensitivity of teak shoots to the negative pressure treatment was tested. ***In vitro*** cultured teak shoots of the ITB, P97 and P108 clones were used as representatives to test the shoot sensitivity to the vacuum-infiltration treatment. Vacuum-infiltration was carried out under sterile condition using a dessicator, in which open-capped culture vessels with sterile teak shoots planted on solid medium were placed. Shoots cultured in the culture-vessels were submerged in liquid MS medium and submitted to vacuum at -80 kPa for 5 and 10 min. To study the effect of negative pressure treatment to the viability of shoots, the shoot freshness was observed until 14 days. The viable condition was indicated as fresh, vigorous, and healthy performance of shoots. The percentage (%) of shoot viability was defined as the numbers of viable shoots per total numbers of evaluated shoots x 100%. Each treatment consisted of 10 replications of culture vessels with 3-5 randomly allocated shoots per vessel. Experiments were repeated twice. Data were analyzed using Duncan's New Multiple Range Test (DNMRT).

In transformation experiments, the suspension culture of ***A. tumefaciens*** LBA4404 containing pBI.121 (OD_{600nm} = 0.6-0.8) was poured into culture vessels, which contained teak shoots cultured on solid culture medium, until all teak shoots were fully submerged and then vacuum infiltrated for 5 min. In parallel to infiltration with ***A. tumefaciens*** LBA4404 containing pBI.121, equal numbers of teak shoots was infiltrated with ***A. tumefaciens*** LBA4404 without binary plasmid and some others were vacuumed in antibiotic-free YEB liquid medium only without ***A. tumefaciens***.

After discarding the bacterial suspension from culture vessels, infiltrated shoots were co-cultivated in dark condition at 25 ± 2ºC.

Table 1. Recovery and viability (%) of shoots from representative teak clones, i.e. the ITB, P97 and P108 after being vacuumed at -80 kPa with various period of vacuuming duration.

Teak clones	Vacuuming duration (min)	No. of Shoots tested	Shoot viability* (%)
ITB	0	60	100[a]
	5	60	76.7[b]
	10	60	33.3[c]
P97	0	80	100[a]
	5	78	75.6[b]
	10	80	28.8[d]
P108	0	60	100[a]
	5	62	77.4[b]
	10	65	29.2[d]

*The percentage of shoot viability was defined as the numbers of viable shoots per total numbers of evaluated shoots x 100%. Each treatment consisted of 10 replications of culture vessels with 3 to 5 randomly allocated shoots per vessel. Experiments were repeated twice. Values followed by the same letters are not significantly different at the 0.05 level as determined by Duncan's Multiple Range Test (DMRT).

Three days after co-cultivation, inoculated shoots were removed from culture vessels and cleaned from the agar medium that attached to the basal end. To eliminate the excess of residual bacteria, inoculated shoots were washed 4-5 times with sterile distilled water, immersed in 200 mg/l carbenicillin solution for 5-10 min, and plotted to dry on a sterilized filter paper. To prevent the growth of bacteria that were penetrated in sub-epidermal layers of organ tissues, cleaned inoculated shoots were re-planted on selective culture (SC) medium containing 200 mg/l carbenicillin and 50 mg/l kanamycin. After seven days on SC medium, selected putative transformed (PT) shoots were randomly collected for histochemical GUS analyzing.

Evaluation of GUS activity

The evidence of GUS activity in shoot tissues was used to express the occurrence of genetic transformation in inoculated teak shoots. The evaluated shoots were 1) collected putative transformed (PT) shoots, which were inoculated with ***A. tumefaciens*** LBA4404 containing pBI.121, 2) shoots that were inoculated with strain LBA4404 without the binary vector, 3) shoots that were vacuumed in YEB medium without ***A. tumefaciens,*** and 4) shoots that were not inoculated (non-inoculated shoots). We also tested the GUS activity in bacteria cultures to determine the possible cryptic expression of GUS within the bacteria.

The GUS activity was confirmed using the histochemical GUS assay as described by Jefferson et al. (1987). All evaluated shoots were immersed and vacuum infiltrated in 100 mM phosphate buffer containing 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-glucuronide), 10 mM EDTA and 0.3 M mannitol for 5 min, and incubated at 37^{o}C for 24 h. The dark blue color of dichloro-dibromo-indigo (ClBr-indigo), observed in shoot tissues, was used as the indication of GUS activity. The GUS expression was observed under a binocular microscope and recorded with a Nikon photograph camera attached to the microscope.

Shoots with GUS activity were evaluated to determine the transient transformation efficiency. The percentages of shoots with GUS positive was defined as the numbers of GUS positive shoots per total numbers of evaluated shoots x 100%. From all GUS positive shoots, the frequency (%) of each shoot organ, i.e. shoot-tip, leaf and stem, showing GUS activity was also calculated. Experiments were arranged as a complete-randomized-block design. Each treatment consisted of 15-20 replications of culture vessels with 4-6 randomly allocated shoots per vessel.

RESULTS AND DISCUSSION

Sensitivity to vacuum-infiltration

Preliminary experiments showed that teak shoots of representative teak clones, i.e. the ITB, P97 and P108, were performing a negative response to the pressure treatments at -80 kPa. Vacuuming treatments seemed to induce stress and fade shoots. After vacuuming for 5 min, the wilted teak shoots were mostly back to its healthy and fresh performance in 2-3 days. However, the percentages of shoot viability after vacuuming for 5 min were different among teak clones (Table 1) i.e. 76.7% for the ITB clone, 75.6% for the P97 clone, and 77.4% for the P108 clone.

Vacuuming shoots for 10 min raised numbers of wilted shoots to more than 50% and caused necrotic responses in shoot tissues especially in young leaves and shoot-tips. When the brownish-necrotic tissues covered more than 50% of shoot organs it subsequently led to shoot death in 14 days. The necrotic effect was probably due to the hypersensitive response of teak shoot-tissues to the negative pressure of vacuum infiltration treatment and more likely to occur in young tender tissues. Lengthening the duration of vacuuming until 10 min significantly decreased the percentages of shoot viability i.e. 33.3% for the ITB clone, 28.8% for the P97 clone, and 29.2% for the P97 clone (Table 1).

The use of vacuum-infiltration technique in this study was proposed to enhance bacterial cell penetration into the cell layers beneath the epidermis of teak shoots. Charity et al. (2002) found that vacuum-infiltration allow-

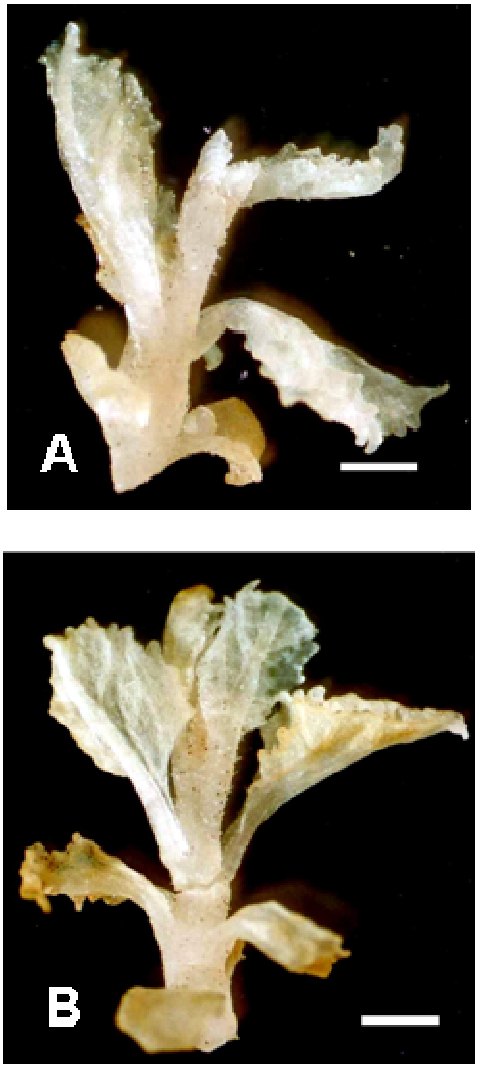

Figure 2 A-B. Teak shoots with no GUS activity. Bars = 2 mm.

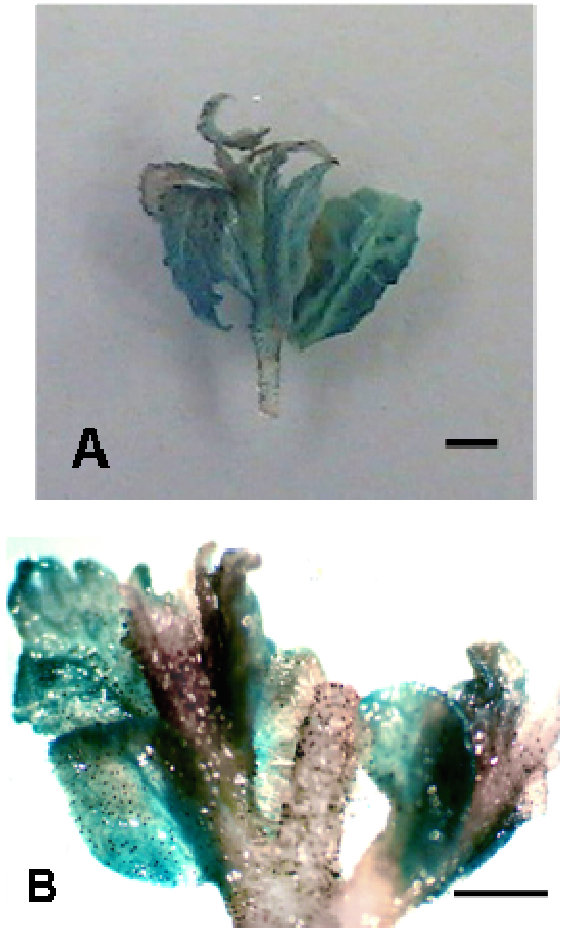

Figure 3 A-B. Teak shoots with transient GUS expression, indicated with the dark-blue stained tissues in various parts i.e. shoot-tips, leaves, petioles, partly in stems. Bars = 2 mm.

ed ***Agrobacterium*** penetrating several layers deep through the sub-epidermal layers of cotyledon and apical merismatic dome tissues of pine. However, to avoid the hypersensitive response of teak shoot tissues to the negative pressure treatment, based on the result of our preliminary experiments, the vacuum-infiltration was conducted for not longer than 5 min in all further transformation experiments of teak.

Evidence of GUS expression

The pBI.121 binary plasmid is containing the uninterrupted GUS gene that could cause the low level of cryptic expression within the bacteria itself (Zaldívar-Cruz et al., 2003). To determine the possible occurrence of cryptic expression within the bacteria we tested the GUS activity in bacteria cultures. The result showed that there was no detectable GUS activity in bacteria cultures.

After seven days of culturing on selective culture (SC) medium containing 50 mg/l kanamycin and 200 mg/l carbenicillin, most of the vacuum-infiltrated teak shoots were performing healthy and fresh condition. The GUS activity was evaluated from randomly selected putative transformed (PT) shoots that performed normal and healthy growth. No GUS activity was detected in either non-inoculated shoots, or in shoots that were inoculated with strain LBA4404 without the binary vector, or in shoots that were vacuumed in YEB medium without A. tumefaciens (Figure 2A-B). Transient gus expression was detected in GUS positive shoots by the evidence of dark-blue stained tissues which mostly covered 20 to 80% of the shoot-parts i.e. shoot-tips, leaves, petioles, and stems (Figure 3A-B). The confirmation of GUS activity indicated the chimeric expression in all selected PT shoots in which the GUS positive and negative tissues were both detected in the same shoot. In some teak shoots, the blue staining was diffuse.

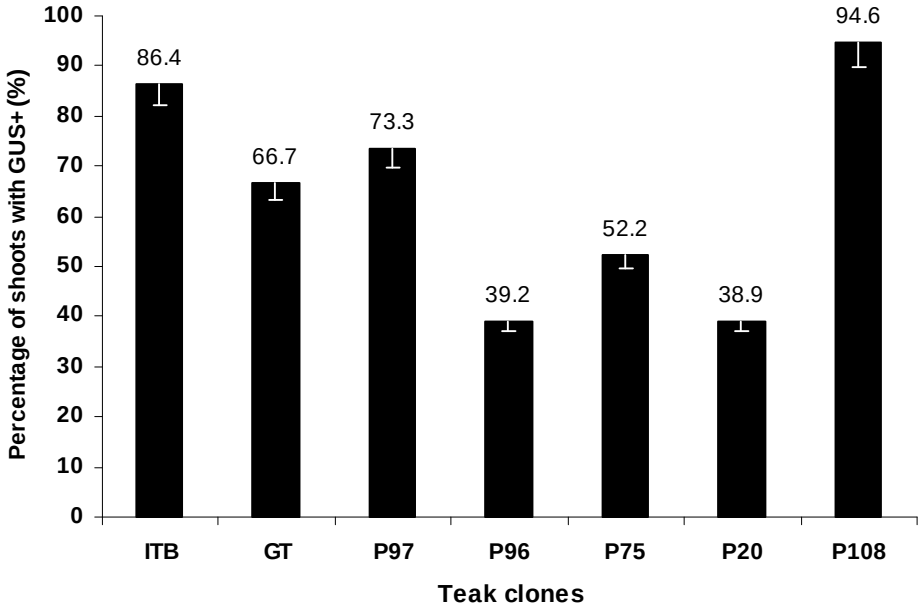

Figure 4. The percentages (%) of inoculated shoots with GUS positive of various teak clones, which was defined as numbers of GUS positive shoots per total numbers of evaluated shoots x 100%. The number on top of each bar represents the value of TE of each teak mother plant. Error bars indicate ± SE at $p = 0.05$.

Effects of genotypic variation

Based on the evidence of GUS activity in teak shoot tissues, the result indicated that ***A. tumefaciens*** strain LBA4404 carrying the pBI.121 was compatible with shoot explants of all tested teak clones. However, based on the percentages of shoots with GUS positive per total numbers of evaluated shoots, the transient transformation efficiencies (TEs) of various teak clones varied (Figure 4). The highest TE (94.6%) was observed from the P108 teak clone and the lowest TE (38.9%) was from the P20 teak clone. Apparently, ***A. tumefaciens*** strain LBA4404 was also highly effective for transformation of the ITB (TE_{ITB} = 86.4%), P97 (TE_{P97} = 73.3%), GT (TE_{GT} = 66.7%), P75 (TE_{P75} = 52.2%) clones as more than 50% of evaluated shoots were transiently detected GUS positive. On the contrary, strain LBA4404 was likely less effective for transformation of the P20 and P96 clones as the TEs of those clones (TE_{P20} = 38.9%; TE_{P96} = 39.2%) were below 50% and much lower than those of other clones.

It seemed that genotypic variation among the teak clones had caused the difference of host-plant response to bacterial interaction and evoked the distinction in transformation efficiency. Elite teak clones were selected from natural population of teak trees that grow in teak forest plantation managed by Perum Perhutani, the Indonesian State Forest Enterprises Co. We had studied the genetic variability among selected elite teak clones (Widiyanto et al., 1998). More than 100 teak clones, including the P20, P75, P96, P97, P108 and the ITB (as a sample of a non-elite clone), were analyzed using Random Amplified Polymorphism DNA markers (RAPD) to evaluate the genetic relationship among clones. From the dendogram illustration we investigated that P20, P75, P96, P97, P108 and the ITB were not from the same genetic cluster. The results of molecular analyses showed the evidence of genetic variation among those teak clones.

Many studies also indicated that the compatibility between bacterial strain and plant-host tissues was obviously genotype specific. ***A. tumefaciens*** strain LBA4404 containing the pBI.121 binary vector was known to be highly effective for transformation in ***Pinus radiata*** embryos (Cerda et al., 2002), annatto hypocotyls (Zaldívar-Cruz et al., 2003), and Himalayan poplar petioles (Thakur et al., 2005). However, the LBA4404 strain with pBI.121 binary vector was less effective in transformation of ***Capsicum*** spp. (Ochoa-Alejo and Ramirez-Malagon, 2001). In another study, Lee et al. (2005) used two genotypes of perilla (***Perilla frutescens***), Yeupsil and Daesil, in their transformation experiments using ***A. tumefaciens*** strain EHA105 which harbours binary plasmid pBKI or pIG121Hm. The result showed that only Yeupsil genotype was successfully transformed. The effect of genotype in transformation efficiency was also studied in ***Agrobacterium***-mediated transformation of two elite aspen hybrid clones (Dai et al., 2003). They reported that some modifications of procedures had to be made to suit each genotype.

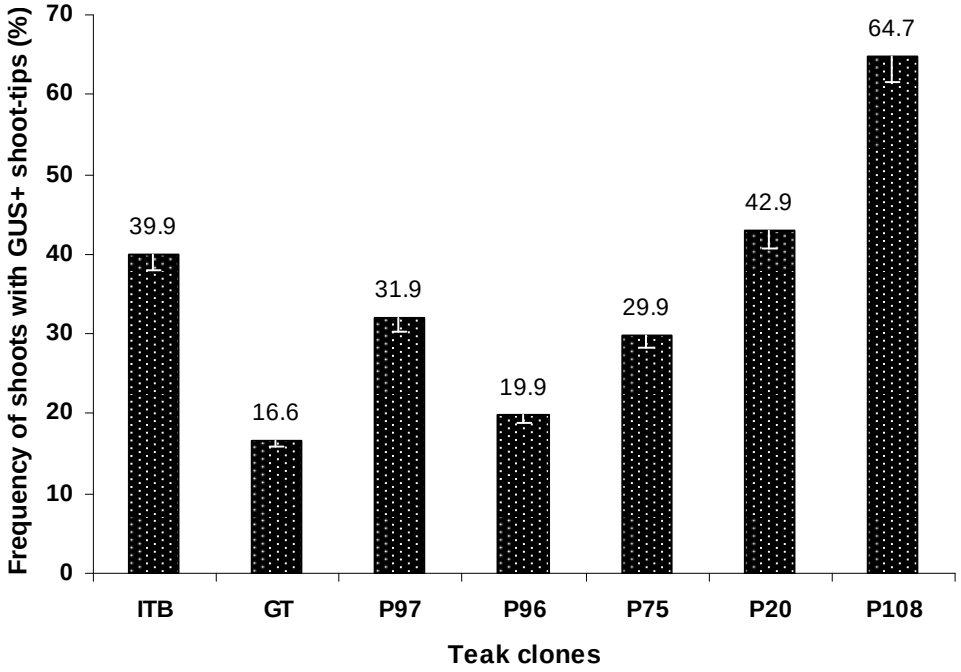

Figure 5. The frequency (%) of shoots with GUS positive in shoot-tips of various teak clones, which was defined as numbers of shoots with GUS positive shoot-tips per total GUS positive shoots x 100%. The number on top of each bar represents the value (%) of each teak mother plant. Error bars indicate ± SE at ***p*** = 0.05.

Effects of organ and tissue specific

Further observation showed that ***Agrobacterium***-mediated transformation of teak shoots was also organ and tissue specific. Our results showed that the evidence of the dark blue color of ClBr-indigo varied among various tissues of shoot organs (Figure 3A-B). In some shoots, the GUS activity was expressed homogenously over large areas whereas in other shoots the blue color of GUS activity was localized only in specific-organ tissues i.e. shoot-tips, leaves, petioles or stems.

Generally, the GUS staining was strongly detected in older tissues of stems or leaves of teak shoots whereas young tissues of shoot-tips exhibited a lower expression of GUS activity (Figures 5 - 7). The evidence of GUS positive in the shoot-tips was highly detected in the P108 teak clone which reached 64.7% of its total GUS positive shoots and the lowest frequency (16.6%) was observed in the GT teak clone (Figure 5). The highest frequency of GUS positive in stem was found in teak shoots of the P96 teak, which was 100% of its total GUS positive shoots and the lowest frequency was observed in the P97 teak with only 48% of its total GUS positive shoots was GUS positive in the stem-tissues (Figure 6). Compare to the shoots with GUS positive in the stem-tissues, the frequency of shoots with GUS positive in leaf-tissues was mostly lower (Figure 7). High frequencies of shoots with GUS positive in leaf-tissues were observed in tissues of the ITB (95.3%), P108 (82.2%), P97 (78.9%), GT (66.7%), and P75 (51.2%) teak-shoots as more than 50% of its total GUS positive shoots was positive in the leaf-tissues. The low frequencies of shoots with GUS positive in leaf-tissues were the P96 (39.5%) and P20 (14.7%) teak-shoots.

Many studies showed a similar result. In many cases, tissues of older plant organs, i.e. stem, expanded-leaves, petioles, etiolated-hypocotyls or roots usually exhibited a strong GUS expression as the result of transformation. However, the influence of organ and tissue specific in compatibility of ***Agrobacterium*** with plant-host tissues varied depending on the plant species. In ***Agrobacterium***-mediated transformed sweet orange and citrange, the evidence of GUS staining was very strong in root, stem and leave tissues (Yu et al., 2002). In perilla (***P. frutescens***), of the three types of explants i.e. hypocotyl, cotyledon and leaf explants that were used in ***Agrobacterium***-mediated transformation experiments, the hypocotyl explants resulted in the highest transformation efficiency (Lee et al., 2005). In cyclamen (***C. persicum***), younger hypocotyl-tissues (63 days old) had a higher transformation efficiency than that of the older (101 days old) hypocotyl-tissues (Boase et al., 2002). The results indicated that we should take the effects of genotype, organ and tissue specificity in compatibility into our consideration when developing procedure of ***Agrobacterium*** mediated transformation.

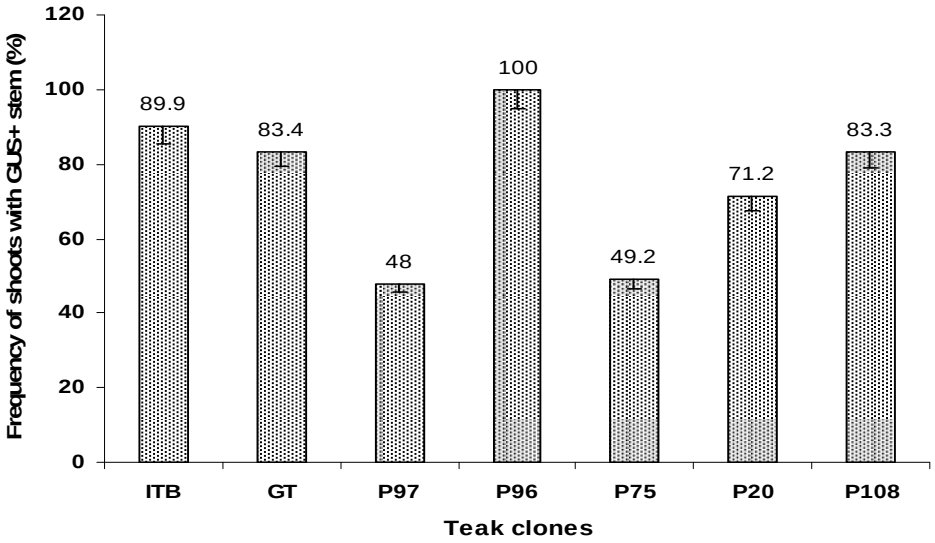

Figure 6. The frequency (%) of shoots with GUS positive in stem-tissues of various teak clones, defined as numbers of shoots with GUS positive stems per total GUS positive shoots x 100%. The number on top of each bar represents the value (%) of each teak mother plant. Error bars indicate ± SE at $p = 0.05$.

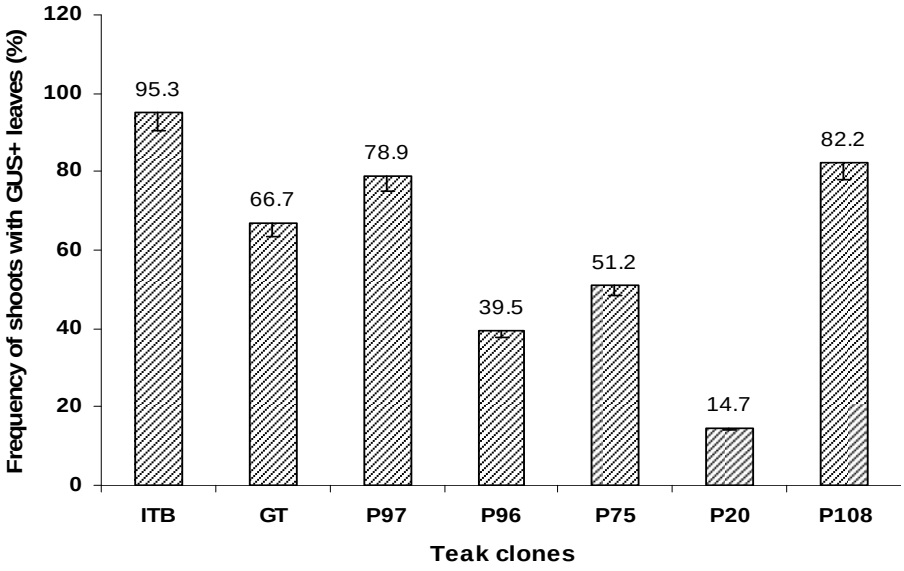

Figure 7. The frequency (%) of shoots with GUS positive in leaf-tissues of various teak mother plants, defined as numbers of shoots with GUS positive leaves per total GUS positive shoots x 100%. The number on top of each bar represents the value (%) of each teak mother plant. Error bars indicate ± SE at $p = 0.05$.

Growth of selected transformants

After a three-week period of culture on selective culture (SC) medium containing 50 mg/l kanamycin and 200 mg/l carbenicillin, selected putative transformed (PT) shoots were growing and performing normal growth (Figure 8). However, due to the possible chimeric expression in selected PT shoots, some shoots exhibited a very limited growth performance. Compared to those of the control-treatment shoots i.e. the non-inoculated shoots, or shoots that were inoculated with strain LBA4404 without the binary vector, or shoots that were vacuumed in YEB medium without ***A. tumefaciens***, the PT shoots showed healthier and no indication of brownish-necrotic tissues of shoots (not shown). Further studies have been in progress to observe the growth and ***in-vitro*** regeneration

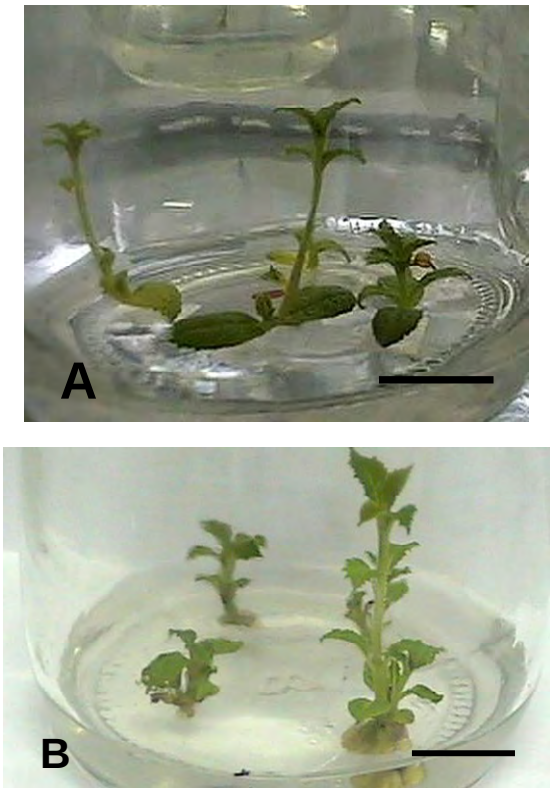

Figure 8 A-B. Some selected putative transformed (PT) shoots cultured on selective culture medium containing 50 mg/l kanamycin and 200 mg/l. carbenicillin after a three-week period of culture. Bars = 10 mm.

capability of transformed teak-shoots.

Concluding remarks

Hence we demonstrated that shoot-tissue cultures can be used as the target explants in ***Agrobacterium***-mediated transformation of teak. The transient transformation efficiency varied among teak clones indicating that the compatibility of ***A. tumefaciens*** strain LBA4404 with teak shoot explants was genotype-specific. Also, the evidence of GUS activity was localized mostly in specific-organ tissues of teak shoots expressing the influence of organ-tissue specific in bacterial and plant-host interaction. This preliminary study is very important to initiate the application of ***Agrobacterium***-mediated transformation procedure in teak tissues. For future experiments, the use of other strains of ***A. tumefaciens*** should become a consideration. To our knowledge, this is the first report of ***Agrobacterium***-mediated transformation of teak using shoot-tissues as the target explants.

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