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The effect of *Agrobacterium* densities and inoculation times on gene transformation efficiency in rubber tree

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Rubber tree belonging to the genus *Hevea* is an economically important crop of Thailand and South-east Asia. To optimize its agronomical trait for glyphosate-resistant, *in vitro* gene transformation through *Agrobacterium tumefaciens* was investigated. The bacteria carrying plasmid pCAMBIA 1304, harboring *gus* as screenable marker genes and *EPSPs* gene was used. The shoot tips were immersed in *A. tumefaciens* suspension at optical densities (OD_{600}) at 0.3, 0.6 and 0.9 for various times (15, 30 and 60 min). The results revealed that shoot explants immersed in *A. tumefaciens* suspension at OD_{600} of 0.6 for 30 min gave the higher survival rate after being cultured on glyphosate containing MS medium for one and half months. Assessment of transformed shoots revealed positive results in GUS histochemical assay. The presence of the *gus* and *EPSPs* genes in transformed rubber tree were confirmed by polymerase chain reaction (PCR) technique, dot blot hybridization and Southern PCR hybridization. Specific primers for the *gus* and *EPSPs* genes were designed to amplify a 919 and 1,600 bps DNA fragment, respectively.

Key words: Transgenesis, glyphosate, inoculation time, *Agrobacterium* density, *Hevea.*

INTRODUCTION

Hevea brasiliensis Muell. Arg. belongs to the family Euphorbiaceae originated in south America primarily in the Amazon basin, but it is now mainly cultivated in south-east Asia countries. Rubber (cis-1,4-polyisoprene) is created in over 2,000 species of plants distributed along with 300 genera from seven families (Priya et al., 2006), nevertheless there are only one species of plant for the industrial raw material of natural rubber which is *H. brasiliensis*. Natural rubber has high performance properties that cannot be easily replaced by synthetically produced polymers. Consequently, rubber tree is one of the most commercial sources, and the financial records for 42% of the rubber consumed worldwide (Lardet et al., 2011). Conventional breeding and selection method of any crop species, one of the most important parameters, bring together traits of agronomical interest. In case of rubber tree, genetic improvement has been very slow and time-consuming as the major limitations because of narrow genetic base, non-synchronous flowering, low fruit set, long gestation period, heterozygous nature (grafting),

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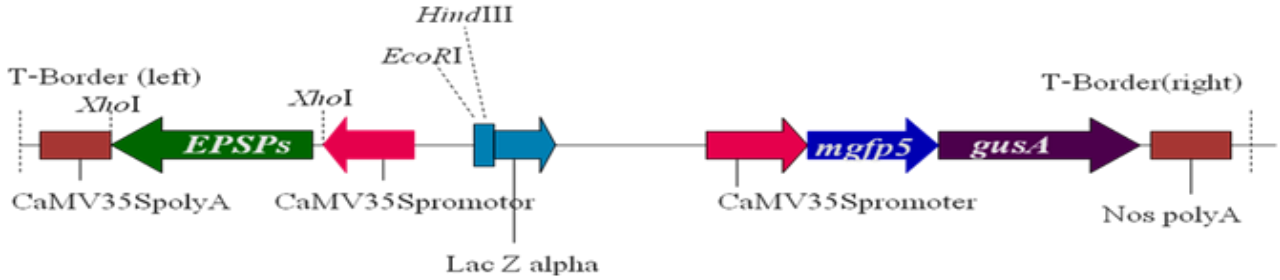

Figure 1. Schematic map of T-DNA region of the binary vector pCAMBIA1304-EPEPs containing the *gus*A gene containing an intron as reporter genes and the *EPSPs* gene conferring glyphosate (*N*-phosphonomethyl glycine) resistance.

insufficient availability of land for field experiments and the absence of fully reliable early selection parameters (Venkatachalam et al., 2007). In addition, breeding program takes up to 25 years for selection and recommend-dation as new clones (Lardet et al., 2011). Its long juvenile phase includes 6-7 years before latex collection.

Genetic transformation offers a potential tool to breeders for adding desirable agronomic traits to crop plants, leading to the development of elite clones in a relatively short period of time (Arokiaraj et al., 2002). *Agrobacterium tumefaciens*-mediated genetic transformation technique has most widely been used for plant species due to its easy protocol without any special equipments. These techniques were developed on numerous *Hevea* clones, GL1, RRII 105 and PB 260 (Arokiaraj et al., 1994; Jayashree et al., 2003; Priya et al., 2006; Montoro et al., 2003; Lardet et al., 2011). Montoro et al. (2003) developed genetic transformation protocol using *A. tumefaciens* in friable integument callus line (clone PB260) for a good frequency of integration of transgenic calli. An efficient genetic transformation procedure was investigated using highly integument-callus lines (Blanc et al., 2006) and GFP selection of transformants (Leclercq et al., 2010).

Nowadays, over-expression of endogenous genes involved in reactive oxygen species scavenging systems, such as MnSOD has been reported (Jayashree et al., 2003). The target of transgene expression in latex cells was also attempted using the promoter from gene *HEV*2.1, which was the major latex Hevein protein (Montoro et al., 2008). Moreover, genetic transformation protocol was developed using the transfer of a synthetic *CP4 EPSPS* transgene, as a conditional positive selectable marker, into commercially relevant zonal pelargoniums using an *A. tumefaciens* strain in combination with a novel step-down glyphosate selection system. Glyphosate is a commercial herbicide used in the control of weed species which exerts its action on plants through inhibition of EPSPS.

This chemical is not detoxified, and consequently here is no cross-protection afforded to evolve resistant weeds (Chen et al., 2012).

In addition, glyphosate is a highly mobile selection agent and translocates throughout the plant and so is less dependent on direct contact of the target tissue than some other selection agents (Howe et al., 2002). However, transformation efficiency was depended on many factors, protocols and cultivar-dependent. Until now there is no report available regarding the gene transformation of glyphosate-resistant shoot of rubber tree.

The introduction of foreign genes into plants was assessed in the transformants by PCR analysis and Southern hybridizations. The process of genetic transformation in *H. brasiliensis* using *A. tumefacients* for glyphosate resistant rubber was optimized.

MATERIALS AND METHODS

Plant material

Seeds from a indigenous clone of rubber tree, naturally grown at Prince of Songkla University, Hat Yai campus, Songkhla province, Thailand, were collected and used as explants for zygotic embryo culture. After 2 weeks of culture, seedlings were obtained and they were excised into two parts, shoot tip and hypocotyl node. The two types of explants were cut into 1.5-2 cm in length and cultured on shoot induction medium (SIM) supplemented with 5 mg/l 6-benzyladenine (BA), 3% sucrose and 0.05% activated charcoal as reported by Te-chato and Muangkaewngam (1992).

The medium's pH was adjusted to 5.7 with 0.1 N HCl or KOH before adding 0.75% agar and autoclaved at 1.05 kg/cm^2, at 121°C for 15 min. The cultures were maintained at 28±0.5°C under fluorescent lamps at light intensity of 12.5 µmol/m^2/s; 14 h photoperiod for 1 month. Single shoot at 1 cm was excised and used for transformation.

Bacterial plasmid

A. tumefaciens strain EHA105 containing the plasmid pCAMBIA1304-EPEPs which harbored ß-glucuronidase (*gus*) and 5-enolpyruvylshikimate-3-phosphate synthase (*CP4 EPSPs*) genes (Figure 1) was used in this study. A single colony of this bacteria was pick out and suspended in 25 ml liquid LB medium (10 g/L tryptone, 5 g/L Bacto yeast extract, 5 g/L NaCl, pH 7.0) containing 50 mg/L kanamycin and incubated on a rotary shaker at 100-150 rpm in the dark at 28°C. After proliferation in LB medium overnight, the cells were collected and resuspended in SIM. The *A. tumefaciens*

suspension was adjusted by spectrophotometer at optical density (OD) of 600 nm and used for transformation.

Agrobacterium densities and inoculation time

The shoot tips were immersed in 25 ml of the *A. tumefaciens* suspension at optical density (OD_{600}) at 0.3, 0.6 and 0.9. The cultures were maintained on rotary shaker in darkness at 100 rpm at 28°C for 15, 30 and 60 min. The explants were placed on sterile tissue papers before transfer to co-cultivation medium which was shoot induction medium (SIM) containing 200 µM acetosyringone. The co-cultivation was kept in the dark at 28°C for 3 days. After co-cultivation, the explants were washed with liquid SIM containing 400 mg/L cefotaxime for 10 min to remove excess bacteria. Then, explants were transferred to SIM supplemented with 200 mg/L cefotaxime to eliminate bacteria for 2 weeks. The inoculated explants were then transferred to selective medium (SIM containing 0.5 mM glyphosate). After 2 weeks of culture, inoculated explants were cultured on SIM containing 2 mM glyphosate for early screening of transformed tissues and subcultured every 2 weeks. The cultures were maintained under 12.5 µmol/m^2/s illumination; 14 h photoperiod at 26±2°C.

Histochemical GUS assay and selection of putative transformants

GUS assays were carried out using protocols described by Jefferson et al. (1987). GUS expression was observed by immersing inoculated explants in X-gluc buffer consisting of 2 mM X-gluc, 100 mM sodium phosphate buffer pH 7.0, 0.5 mM potassium ferricyanide, and 0.5 mM potassium ferrocyanide. The explants were incubated overnight at 37°C in the dark, and washed with absolute methanol for overnight. The percentage of *gus* expression which was the blue percentage per explant, was recorded and scored under stereomicroscope.

For selection, glyphosate was used for selection of putative transformants. Non-inoculated and inoculated shoots were cultured on selective medium (SIM supplemented with 2 mM glyphosate). After 1 and half months of inoculation, the percentage of glyphosate-resistant shoots [% resistant = (number of survival shoots / total number of shoots) x 100] was recorded.

Molecular analysis of the transformed plantlets by PCR analysis, dot blot hybridization and Southern blot PCR hybridization

Genomic DNA was isolated from young leaf (0.05 g) of non-transformed and transformed plantlets after 1 and half months of culturing on selective medium by the CTAB method (Doyle and Doyle, 1990). The *gus* gene fragment was amplified using forward primer sequence F-primer 5'-CTGCGACGCTCACACCGATAC-3' and reverse primer sequence R-primer 5'-TCACCGAAGTTCATGCCAGTCCAG-3'. The forward and reverse primer sequences for the *EPSPs* gene amplification were 5'-CCATTCCGCTCGAGATGGCACAAATTAACAACATGGC-3' and 5'-ATCCACCGCTCGAGCGGTCATCAGGCAGCCTTCGTAT-3', respectively. The reaction mixture contained 1 µl of genomic DNA (20 ng), 0.5 µl of each primer (5 pmol), 4 µl of dNTP mix (1 mM each), 2 µl ml of PCR buffer, 0.1 µl of *Taq* DNA polymerase (1 U/ml) which was mixed together and adjusted to 20 µl with sterile distilled water. The PCR reaction started at 96°C for 2 min, followed by 30 cycles of denaturation (96 ^{o}C, 20 s), annealing (55°C, 1 min) and extension (72°C, 2 min), with a final extension of 5 min at 72°C. PCR amplified products were separated in 1.0% agarose gel with ethidium bromide and visualized by gel documentation at 260 nm of UV.

For dot blot hybridization, 4 µg of the genomic DNA of non-transformed and transformed plantlets and 2 µL of PCR products were dropped on a nylon membrane (hybond-N, Amersham). Blotted membranes were dried by incubation at 80°C for 1 h. The membranes were pre-hybridized in hybridization solution (5X SSC, 0.1% N-lauroylsarcosine, 0.02% SDS and 1X blocking solution) for 1 h at 65°C. Hybridization was performed with DIG-labeled DNA probe (*gus* or *EPSPs* gene) overnight at 65°C, which was generated using the PCR DIG Probe Synthesis Kit (Roche Applied Science). Hybridized membrane was washed twice in low stringency buffer (2X SSC, 0.1% SDS) for 15 min, twice in high stringency buffer (0.1X SSC, 0.1% SDS) for 15 min and once in washing buffer (1X maleic acid buffer, 0.3% tween 20) for 10 min. The membrane was blocked in blocking solution (dilute 10X blocking solution 1:10 with maleic acid buffer) for 30 min. After that, the anti-digoxigenin conjugate alkaline phosphate was added into blocking solution and incubated for 30 min. The membrane was then transferred to detection buffer (0.1 M Tris-HCl, 0.1 M NaCl) for 3 min at room temperature. Finally, the membrane was dropped by chemiluminescent substrate (CDP star^{TM}) and exposed to Kodak BiomaX-Omat film for autoradiography. The film was washed with developer and fixer solution after exposure in the cassette for 60 min.

For Southern blot PCR hybridization, PCR products 15 µL (1-2 mg/µL) were separated by 1% agarose gel electrophoresis. The gel was treated with 0.25 N HCl to depurinate briefly the DNA and then denatured with an alkaline solution for 30 min and neutralized for 30 min. The denatured DNA was then transferred to a nylon membrane (hybond-N, Amersham). Blotted membrane was dried by incubation at 80°C for 1 h. The blotted membrane was hybridized and detected using the same protocol according to dot blot hybridization as describe above.

Statistical analysis

Data were subjected to ANOVA analysis and significance was determined with the balance analysis test with a level of significance at p = 0.05 using statistically analysis system (SAS). Two factorial completely randomized design with three replicated series was used. Each replication consisted of nine samples.

RESULTS AND DISCUSSION

Agrobacterium densities and inoculation times

The explants immersed in *A. tumefaciens* suspension at optical density of 600 nm at various concentration of *A. tumefaciens* (0.3, 0.6 and 0.9) and inoculation times (15, 30, 60 and 90 min) revealed that shoot explants immersed in *A. tumefaciens* suspension at optical density 600 nm of 0.9 gave the highest GUS expression in all parameters tested. In addition, transient GUS activity increased with inoculation time, reaching 57.67% GUS expression for 90 min, significant difference with the other times of inoculation (Table 1 and Figure 2). In this experiment, the effect of inoculation time on GUS expression was not significantly different. However, the effect of *A. tumefaciens* density was significantly different

Table 1. Effect of *A. tumefaciens* densities and inoculation times on transient expression of the *gus* gene (%) in rubber tree after 4 weeks of transformation.

***Agrobacterium tumefaciens* densities**	**Transient expression of the *gus* gene (%)**			
	Inoculation times			
	15 min	**30 min**	**60 min**	**Mean density**
0.3	35.33^{c}	37.67^{bc}	38.33^{bc}	37.11^{B}
0.6	40.47^{abc}	46.67^{abc}	54.33^{ab}	47.22^{A}
0.9	48.33^{abc}	53.67^{ab}	57.67^{a}	53.22^{A}
Mean time	41.44^{A}	46.00^{A}	50.11^{A}	
CV (%)		20.51		
F (Rep)		1.95 ns		
F (Density)		7.46 *		
F (Time)		2.11^{ns}		
F (Density x Time)		0.28^{ns}		

ns = not significant, *p < 0.05 (significant). Means with different small letter indicate significant differences among treatments (p< 0.05) and mean with different capital letter indicate significant differences among treatment combination. The data are the means from nine samples with three replicates.

in which optical density 600 nm of 0.9 gave the highest GUS expression (53.22%). There was no interaction effect between inoculation times and *A. tumefaciens* densities. Nevertheless, the concentration of *A. tumefaciens* affected survival rate of explants (Figure 3). The result showed that glyphosate-resistant shoots died after 1 and half month of culture on selection medium, which was SIM medium containing 0.5-2 mM glyphosate due to high density of *A. tumefaciens* cell (OD_{600}=0.9). This evident caused an overgrowth of *A. tumefaciens* and decreased in the survival rate of plant tissues. For that reason, the highest glyphosate-resistant shoots were obtained from optical density at 0.6. A similar result has also been reported in many plant species, such as alfafa (Zhang et al., 2010) and *Parthenocissus tricuspidat* (Yang et al., 2010). However, inoculation time was not significantly different in percentage of glyphosate-resistant shoots. Significant interaction was not found between inoculation time and *A. tumefaciens* density in glyphosate-resistant shoots.

In the case of inoculation time, the high transient GUS activity was done from 60 min inoculation time to gene transformation. It showed that shoot explants immersed in *A. tumefaciens* suspension for 90 min gave the highest GUS expression in all treatment tested. However, longer period of inoculation decreased the percentage of survival rate of explants co-cultured with *A. tumefaciens*. According to this result, the inoculation period was critical factor for transformation. The highest survival rate of shoots was obtained from 30 min inoculation (Table 2 and Figure 4). Indeed, the inoculation time of *Agrobacterium* has a close relation with penetration or transmission of T-DNA in the plant tissue. The presence of a larger number of bacterial cells might enhance both the number of transformation events and tissue response related to biotic stress. Kondo et al. (2000) reported that the periods of inoculation seem to be effective for the efficient transfer of the T-DNA into plant cells, and longer periods of inoculation gave negative effect on survival rate of explants. The inoculation time usually applied in transformation procedures is about 30 min for immature embryo of oil palm (Abdullah et al., 2005) and alfafa calli (Zhang et al., 2010), 40 min for embryogenic callus of *P. tricuspidata* (Yang et al., 2010), and up to 2 h for tobacco leaf ring (Vinod Kumar et al., 2005). Interestingly, Blanc et al. (2006) reported that successful in transformation process of rubber tree took only one second submerging calli in *A. tumefaciens* suspension. Contrary results were obtained in the present study. Firstly, different explants type was used. In the present study, shoot explants were applied. Organized tissues seem to resist to *A. tumefaciens* solution better than callus, thus time required for inoculation might be longer. Secondly, regenerability of those explants was far different. Callus of rubber was reported to be very sensitive to all stimulants applied *in vitro*, e.g. toxin, colchicine (Te-chato et al, 1995). Plantlet regeneration from callus just after treating with those chemicals was not reported. Therefore, *A. tumefaciens* desity at optical density 600 nm of 0.6 and inoculation time for 30 min could improve transient GUS expression (46.67%) and glyphosate-resistant shoot (48.67%) for gene transformation procedure in rubber tree.

Molecular analysis of the transformed plantlets by PCR, dot blot hybridization and Southern blot PCR hybridization

To prove the presence of the *gus* and *EPSPs* gene in

Table 2. Effect of *A. tumefaciens* densities and inoculation times on glyphosate resistance shoots (%) of rubber tree after 1 and half months of transformation.

***Agrobacterium tumefaciens* densities**	**Glyphosate resistance shoots (%)**			
	Inoculation times			
	15 min	**30 min**	**60 min**	**Mean density**
0.3	33.33^{bc}	36.33^{abc}	35.67^{acb}	35.11^{B}
0.6	48.33^{a}	48.67^{a}	45.67^{ab}	37.56^{A}
0.9	28.33^{c}	25.67^{c}	26.67^{c}	26.89^{C}
Mean time	36.89^{A}	36.67^{A}	36.00^{A}	
CV (%)		18.99		
F (Rep)		0.15^{ns}		
F (Density)		18.34 *		
F (Time)		0.04^{ns}		
F (Density x Time)		0.18^{ns}		

ns = not significant *p < 0.05 (significant). Means with different small letter indicate significant differences among treatments (p< 0.05) and mean with different capital letter indicate significant differences among treatment combination. The data are the means from nine samples with three replicates.

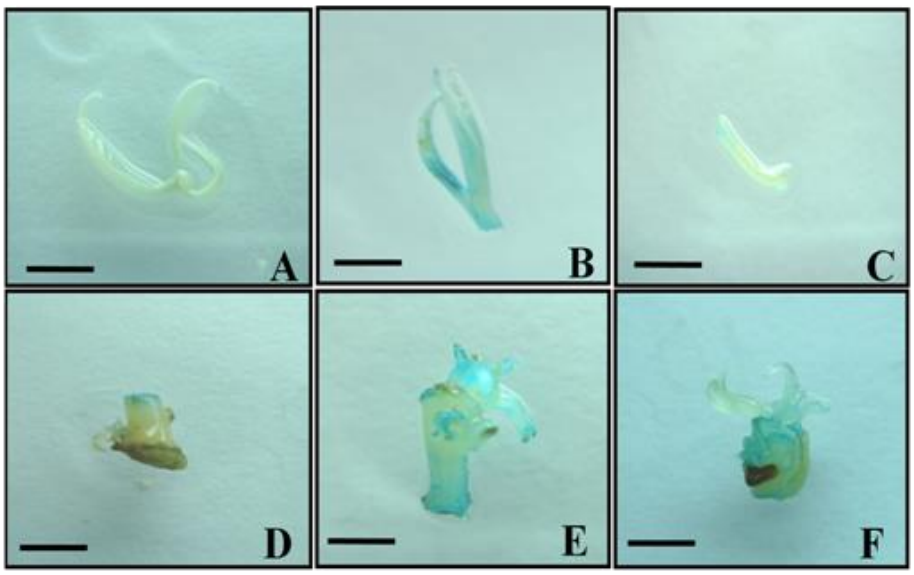

Figure 2. Histochemical assay of β-glucuronidase (GUS) activity in transgenic explants transformed by *Agrobacterium* harboring pCAMBIA1304-EPSPs containing the *gus*A gene and the *EPSPs* gene conferring glyphosate (*N*-phosphonomethyl glycine) resistance (bars= 5 mm). **(A)** Non-transformed leaf. **(B-F)** transformed leaf, petio, stem, shoot and new coming shoot, respectively.

transformed rubber tree, PCR analysis was conducted to evaluate putative transformants, along with non-transgenic plant (negative control). Specific primers for the *gus* gene were designed to amplify a 919 bps DNA fragment. The presence of *gus* genes was confirmed in nine transgenic plants and in the plasmid DNA, whereas the corresponding band was not detected in the non-transgenic control (Figure 5A). For *EPSPs* gene, the transformed plantlets showed the positive results at 1,600 bps of 8 samples from 9 samples (88.89%), but lane number 9 was showed slightly pale band (Figure 5B).

In the case of dot blot hybridization using *gus* gene probe, the genomic DNA of nine samples showed dark color dots indicating the success of gene transfer into plant genome (Figure 6A). On the other hand, dot blot hybridization using *EPSPs* gene showed the positive

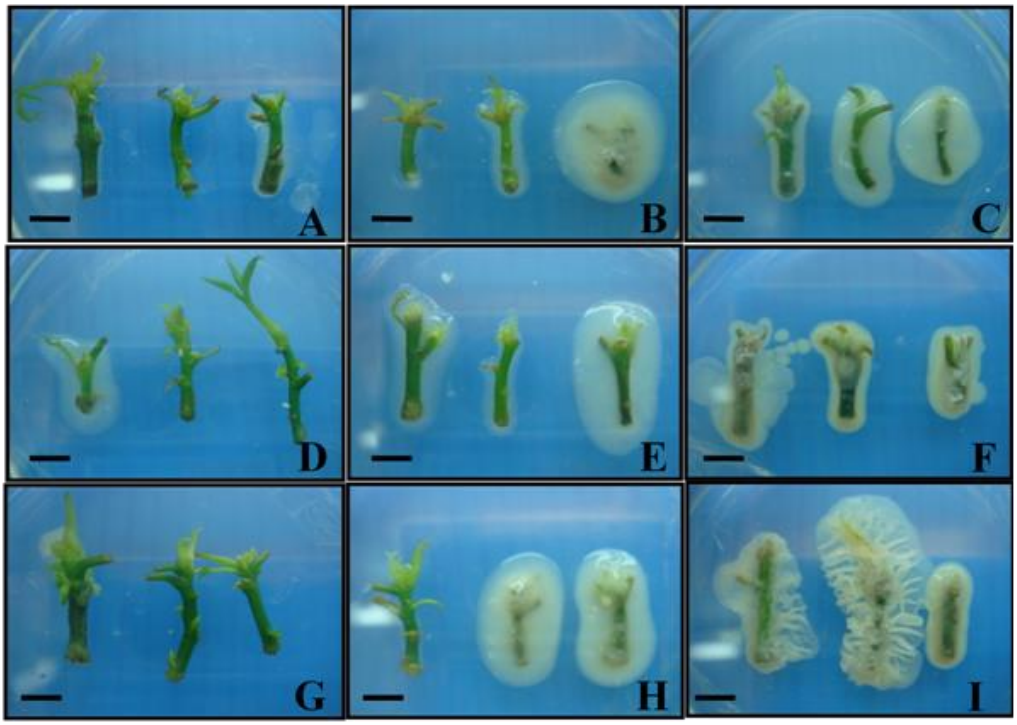

Figure 3. Morphological appearance of shoot explants inoculated with *Agrobacterium* harboring pCAMBIA at various *A. tumefaciens* densities and inoculation times subsequent to culture on co-cultivation medium for 3 days (bars = 5 mm). **(A-C)** Inoculation at OD_{600} = 0.3 for 15, 30 and 60 min, respectively. **(D-F)** Inoculation at OD_{600} = 0.6 for 15, 30 and 60 min, respectively. **(G-I)** Inoculation at OD_{600} = 0.9 for 15, 30 and 60 min, respectively.

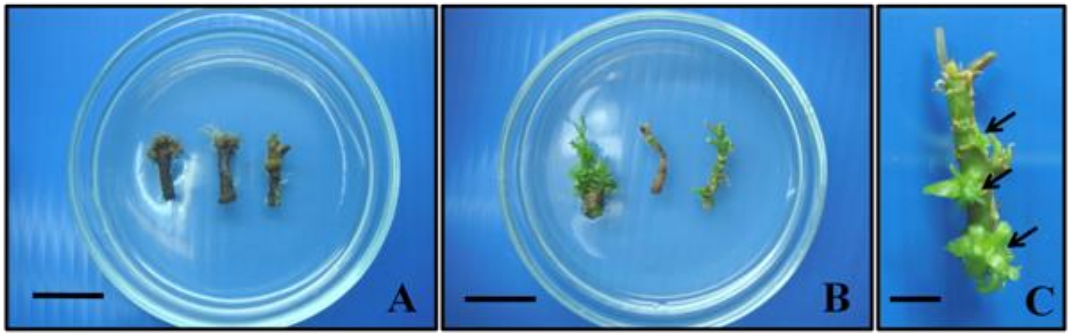

Figure 4. Glyphosate-resistant shoots cultured on selective medium (bar=1 cm). **(A)** Control (non-transformed shoot). **(B)** Shoot inoculated *A. tumefaciens* with OD_{600} = 0.6 for 30 min. **(C)** Newly shoot after culture on selection medium for 1 and haft months.

results of 7 samples from 9 samples (77.78%). The positive transgenic plant samples developed dark black spots as well as the positive control sample, while the non-transformed plantlet samples did not show the dark spots (Figure 6B).

Southern PCR hybridization clearly confirmed the presence of *gus* gene and *EPSPs* gene at sizes 919 and 1,600 bps, respectively. The band of DNA from non-transformed shoot did not appear (Figure 7C). However, Southern PCR hybridization using *EPSPs* gene gave the same result in dot blot hybridization. Only seven positive transgenic plant samples developed dark black bands as well as the positive control samples, while 2 samples and the non-transformed samples did not show the dark bands (Figure 7D). The reason might be due to the transportation of T-DNA from *Agrobacterium* to plant

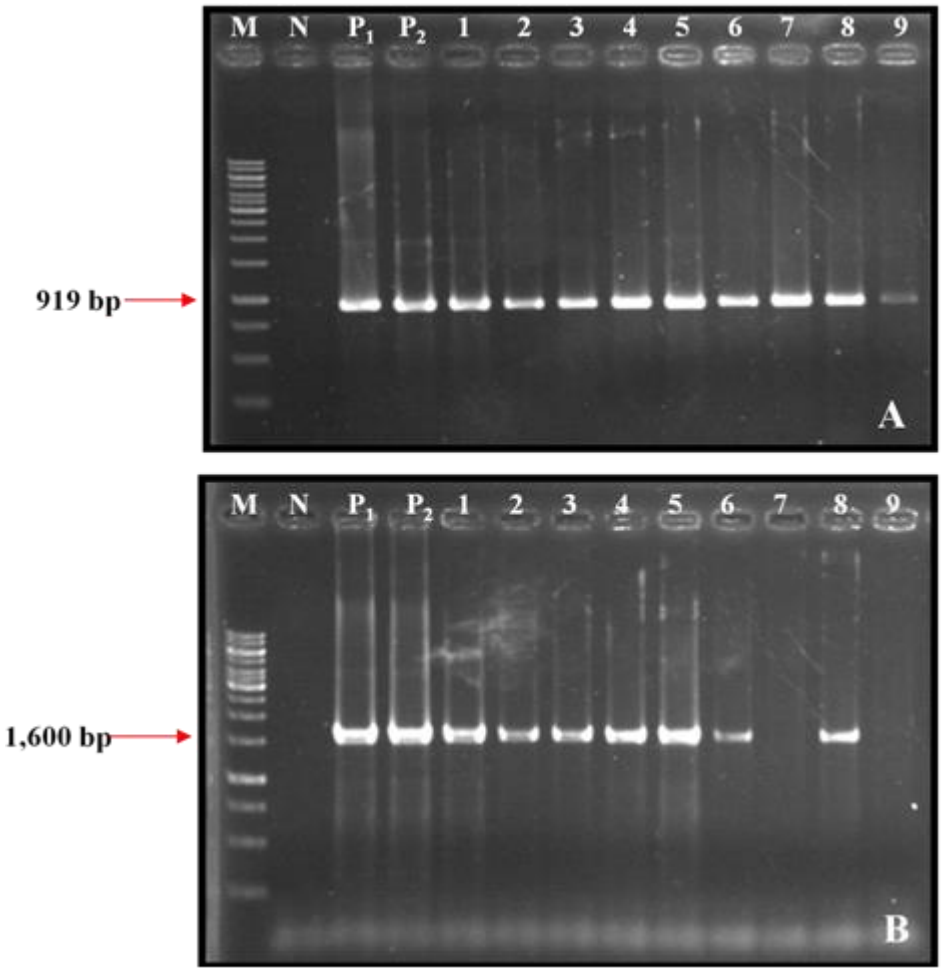

Figure 5. PCR analysis showed the presence of **(A)** *gus* gene at 919 bps and **(B)** *EPSPs* gene at 1,600 bps from different plant genomes. M, marker; N, negative control; P, positive DNA control; 1-9, transformed shoots.

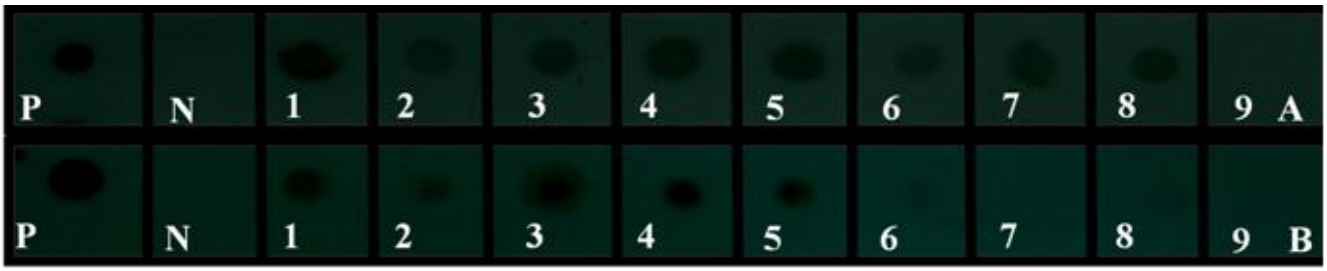

Figure 6. Detection of (A) *gus* gene and (B) *EPSPs* gene in genomic DNA of transformed shoots after 1 and half months on selection medium by dot blot hybridization. P, positive control; N, non-transformed; 1-9, transformed shoots.

genome. Enzyme endonuclease cut T-DNA at right border (RB) from Ti plasmid and inserted RB border into plant genome before left border (LB). Right border connected with *gus* and *EPSPs* genes, respectively. In some case, it is possible that incomplete transfer of T-DNA was performed. For this result, only some reporter genes at the first part was sent to plant genomes while the others were not. Thus, in the present study, all of the transgenic samples presented the *gus* gene, but *EPSPs* gene did not show in some samples.

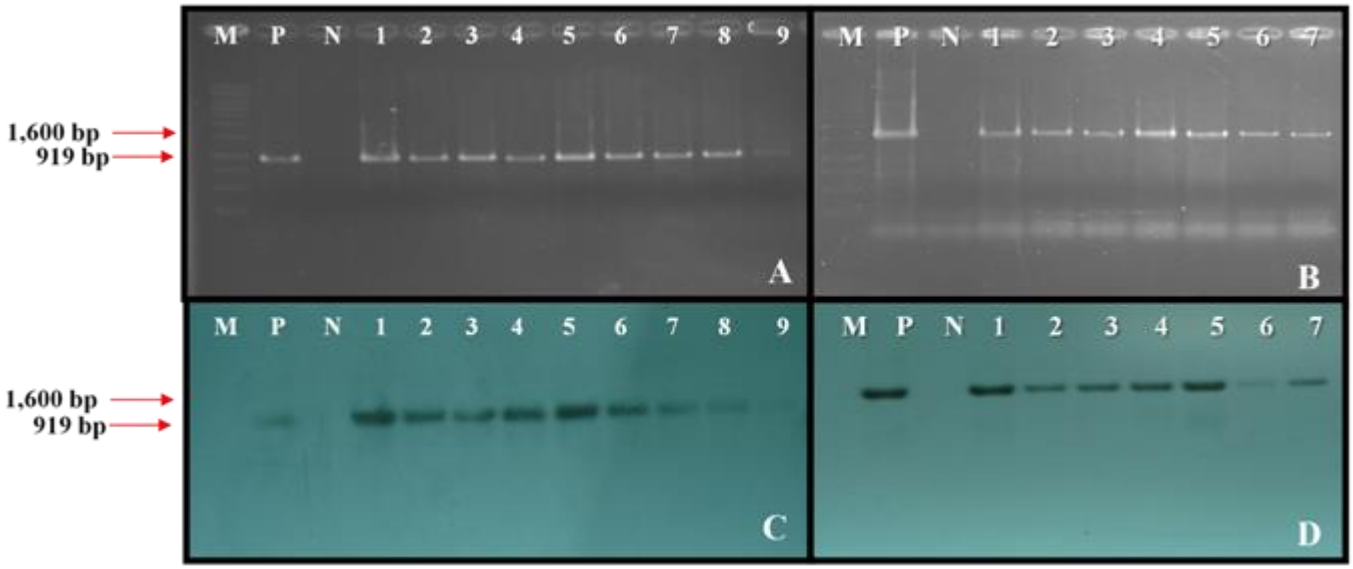

Figure 7. Detection of **(A, C)** *gus* gene and **(B, D)** *EPSPs* gene in transformed shoots after 1 and half months on selection medium by PCR **(A, B)** and Southern PCR hybridization **(C, D).** M, DNA marker; P, positive control; C, non-transformed; N, negative control; 1-8, transformed shoots.

Conclusion

Shoot tip explants inoculated with *A. tumefaciens* carrying plasmid pCAMBIA 1304, harboring *gus* gene as screenable marker genes and *EPESPs* gene for 30 min at optical density of 600 nm to 0.6 gave the highest survival rate of the explants. GUS histochemical assay of transformant revealed positive results. The presence of the *gus* and *EPSPs* genes in transformed rubber tree were confirmed by PCR technique dot blot hybridization and Southern PCR hybridization. Specific fragments of DNA at size of 919 and 1600 bps conferred glucuronidase gene and glyphosate resistance were identified by those techniques.

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Conflict of Interests

The author(s) have not declared any conflict of interests.

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