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Efficiency of Genetic Transformation via Pollen-Tube Pathway of Jatropha (*Jatropha curcas* L.) Based on Histochemical and Molecular Analysis

Efisiensi Transformasi Genetik Tanaman Jarak Pagar (Jatropha curcas L.) melalui Jalur Tabung Polen Berdasarkan Analisis Histokimia dan Molekuler

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ABSTRACT

The genetic transformation via pollen-tube pathway is an alternative method to overcome the constraints imposed by genotype specificity in transformation and regeneration in jatropha (Jatropha curcas L.) tissue culture. Therefore, it is necessary to establish important parameters for efficient genetic transformation of jatropha via pollen-tube pathway. The objective of the research was to study the efficiency of direct transformation of jatropha via pollen-tube pathway based on histochemical and molecular analysis. Solution of purified pCAMBIA1301 DNA plasmid carrying a hptII marker gene and a gus reporter gene with concentration level of 0.05, 0.25, 0.50 µg µl^{-1} were applied to stigma of flowers at 1, 2, 4, 7, 10 h after pollination. Seedling of IP3A, IP3P and JcUMM18 jatropha's genotypes derived from 15 combination treatments of plasmid DNA concentration and application time, also wild type was subjected to histochemical and molecular analyses. Based on those analyses, the efficiency of transformation via pollen-tube pathway of three jatropha genotypes ranged from 1.5-16.7%. PCR analysis showed that a number of positive plants were identified by using specific primers hptII and gus, i.e. 1-3 and 3-7 plants of the 15 combined treatments, respectively. It indicated that the transformation efficiency via the pollen-tube pathway varied in each jatropha genotype.

Keywords: Jatropha curcas L., pCAMBIA1301, plasmid DNA, stigma-drip

ABSTRAK

Transformasi genetik melalui jalur tabung polen adalah metode alternatif untuk mengatasi kendala transformasi dan regenerasi dalam kultur jaringan yang terjadi pada genotipe tertentu tanaman jarak pagar. Oleh karena itu, perlu dilakukan evaluasi parameter-parameter penting untuk transformasi genetik jarak pagar melalui jalur tabung polen yang efisien. Tujuan dari penelitian ini adalah untuk mempelajari efisiensi transformasi langsung dari pohon jarak melalui jalur pollen-tube berdasarkan analisis histokimia dan molekuler. DNA plasmid pCAMBIA1301 murni yang membawa gen marka hptII dan gen pelapor gus dengan tingkat konsentrasi 0,05, 0,25, 0,50 µg µl^{-1} diteteskan pada stigma bunga pada 1, 2, 4, 7, 10 jam setelah polinasi. Kecambah generasi T0 jarak pagar genotipe IP3A, IP3P dan JcUMM18 yang diperoleh dari 15 kombinasi perlakuan dan kontrol (tipe liar) digunakan untuk uji secara histokimia dan analisis molekuler. Berdasarkan hasil analisis, efisiensi transformasi melalui jalur tabung polen dari tiga genotipe jarak pagar berkisar antara 1.5-16.7%. Berdasarkan analisis PCR dengan primer spesifik hptII dan gus, tanaman transgenik diperoleh pada 1-3 dan 3-7 dari 15 kombinasi perlakuan konsentrasi dengan waktu penetesan DNA plasmid. Hal ini mengindikasikan bahwa efisiensi transformasi melalui jalur tabung polen berbeda-beda pada masing-masing genotipe jarak pagar.

Kata kunci: Jatropha curcas L., pCAMBIA1301, plasmid DNA, tetes stigma

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INTRODUCTION

Jatropha (*Jatropha curcas* L., Euphorbiaceae) is a perennial, non-food oilseed and potential biodiesel crop that can be cultivated on marginal land (Costa *et al*., 2010; Madhaiyan *et al*., 2015). Yield variety of jatropha is one of the factor that cause the gap between the potential and actual yields (Djumali and Nurnasari, 2014). Unlike palm oil, soybean, maize, sunflower and other oil crops, there is no widely cultivated improved jatropha varieties. Development of Jatropha, an important tropical biofuel crop, to coastal sandy soil is an urgent situation due to more limited of fossil fuel in one side, and high potency of coastal sandy land that has not been utilized yet (Parwata *et al*., 2014).

Genetic engineering is a promising and feasible way to dramatically improve plant traits, i.e. resistance to plant disease or pest, tolerance to abiotic stress etc. However, application of genetic engineering technique for jatropha breeding is relatively new. Because of the lack of efficient regeneration and transformation procedures, jatropha remains a difficult plant for genetic transformation. Thus, the efficient transformation method for this plant is not fully established yet. One major strategy for producing transgenic jatropha is to improve transformation efficiency (Tsuchimoto *et al*., 2012). Initial transformation experiments of jatropha were conducted by using *Agrobacterium* mediatied. Unfortunately, this procedure produces extremely low transformation efficiency. The step of root induction is also challenging because the resultant rate is usually low (Khemkladngoen *et al*., 2011a,b).

One of the alternative methods to generate transgenic plants in several recalcitrant species is transformation via pollen-tube pathway. This method is used to transfer foreign genes or DNA into zygotic embryos through pollen-tube pathway and then inserting into plant genome (Keshamma *et al*., 2008; Wang *et al*., 2013). Pollen tube pathway-mediated transformation is a tissue culture or genotype independent transformation method and also it does not require expensive instrument (Wang *et al*., 2013). Method of genetic transformation via pollen-tube pathway has been successfully carried out at several plants (Zhang *et al*., 2012; Wang *et al*., 2013), but the application of such methods in jatropha has not been reported yet. This research was aimed to assess the efficiency of direct transformation of jatropha via pollen-tube pathway used plasmid pCAMBIA1301 based on histochemical and molecular analysis.

MATERIALS AND METHODS

This research was conducted in November 2014 until January 2016 at green house and molecular biology laboratory of Center for Agricultural Biotechnology and Genetic Resources Research and Development, Bogor, and at Center for Biotechnology Development, University of Muhammadiyah Malang.

Plasmid pCAMBIA1301 carrying a *hptII* marker gene and a *gus* reporter gene were used in this study. T0 generation plants (the parent transformed plants) of IP3A, IP3P and JcUMM18 genotype derived from 15 treatments of direct transformed via pollen-tube pathway (combination of three concentration level of pCAMBIA1301: 0.05, 0.25, 0.50 µg μL^{-1}; and five time level of stigma-drip: 1, 2, 4, 7, 10 h after pollination) and wild type of each genotipe of jatropha seedlings were subjected to histochemical and molecular analysis. The arrangement of treatments was a randomized complete block design with three replicates. All of seedling that grew well at each treatment was used as a sample.

Hygromycin Resistant Assay

The hygromycin resistant T0 putative transgenic plants were identified by brush-painting method (Bibi *et al*., 2013, modified). The leaf surface of putative transgenic plant was painted with 10 and 20 µl mL^{-1} hygromycin solution (from Hygromycin B solution, 50 mg mL^{-1}, Roche Diagnostics Gmbh) by a small brush and marked with a marker pen to identify brushed leaves with hygromycin solution. The painting was conducted at afternoon. The leaves were scored for hygromycin resistant 48 hours after application. No necrotic symptoms was observed in hygromycin resistant plant. Number of necrosis free plants divided by total number of plants was counted as percentage of hygromycin resistant plants.

Histochemical GUS (β-glucuronidase) Assay

GUS assay was done by incubating the leaf tissues in freshly prepared GUS assay buffer (1g L^{-1} X-Gluc with 0.05M Na_2HPO_4, 0.5mM $K_3Fe(CN)_6$, 0.5mM $K_4Fe(CN)_6$, 10mM EDTA and 0.1% (v/v) Triton X-100) for 12 h at 37 ^oC. Thereafter, the tissues were destained with 70% alcohol to examine and count blue spots. Individual plant with at least one discrete blue region on the leaf were scored as GUS positive (Jefferson, 1987). Number of blue spot plants divided by total number of plants was counted as percentage of positive plants contained gen *gus*.

PCR Analysis

Total genomic DNA of T0 putative transgenic plants were isolated by using modified CTAB method (Dhakshanamoorthy and Selvaraj, 2009). Specific primers for *hptII* (forward 5'-GATGCCTCCGCTCGAAGTAGCG-3' and reverse 5'-GCATCTCCCGCCGTGCAC-3') and gus gene (forward 5'-AACTGGACAAGGCACTACCGG-3' and reverse 5'-GTATCGGTGTGAGCGTCGCAGAAC-3') were used for PCR analysis.. The amplification program for the *hptII* gene was as follows: 94 ^oC for 1 minute for initial denaturation, then 40 cycles at 94 ^oC for 1 minute for denaturation, 58 ^oC for 1 minute for primer annealing, and 72 ^oC for 1 min for extension, followed by a final extention at 72 ^oC for 5 minutes. For the gus gene, the PCR cycle was as follows: 94 ^oC for 1 minute for initial denaturation, then 40 cycles at 94 ^oC for 1 minute for denaturation, 48 ^oC for 1 minute for primer annealing, and 72 ^oC for 1

min for extension, followed by a final extention at 72 °C for 5 minutes. The amplified product were separated in a 1% agarose gel and stained with 10 mg L^{-1} ethidium bromide and visualized under UV gel doc.

Data Analysis

Histochemical and molecular data were subjected to one-way analysis of variance at 5% and 1% level. Before analysis, data would be transformed by √(x+0.5). Duncan's multiple range test at 5% level of significance was used to separate differences between mean of treatments.

RESULTS AND DISCUSSION

Hygromycin Resistant Assay

Hygromycin has been used for the screening of hygromycin resistant plants in various crops (Kumar *et al*., 2013). Selection conditions were optimized for hygromycin resistance in many crops using leaf tip assay method (Noor *et al*., 2000). To minimize the use of hygromycin, this research applied a brush-painting method to screen hygromycin resistant plants with two concentration level of hygromycin, i.e. 10 and 20 µl mL^{-1}. Results demonstrated that Hygromycin-B concentration of 10 µl mL^{-1} could be used to select transgenic plant of jatropha, on the contrary, most of plants that were treated by concentration of 20 µl mL^{-1} experienced necrosis symptoms (Figure 1). Bibi *et al*. (2013) used range of hygromycin (25-200 mg L^{-1}) on cotton leaves at different growth stages. The plants showed healthy and green leaf after treatment (no necrotic symptoms) are considered as transgenic plants.

Results also indicated that necrosis free plants were obtained in almost DNA plasmid concentration and time of stigma-drip treatments in IP3A and IP3P genotype, except combined treatment of 0.05 µg $µL^{-1}$ and 2 h after pollination, 0.25 µg $µL^{-1}$ and 7 h after pollination, 0.5 µg $µL^{-1}$ and 7 h after pollination. In JcUMM18 genotipe, only three combined treatment had necrosis free plants. There were no significant effect of all treatments on percentage of necrosis free plants in each genotipe (Table 1). Percentage of hygromycin tolerance plant of IP3A, IP3P and JcUMM18 genotype range from 1.5-16.7%. Kumar *et al*. (2010) reported that leaf explants of jaropha that had been infected and co-cultivated with *Agrobacterium*, resulted in 3-4 shoots per explant and 20-30% responded positively to hygromycin containing medium. One advantage of use of the brush painting method is not costly and laborious when many plants are used in a hygromycin resistant analysis. However, the method is sometimes affected by environmental or plant conditions. Plant leaves exhibited differential sensitivity to hygromycin application under certain field conditions. The possible reason for this differential sensitivity might be leaf age, young leaves from top canopy being more sensitive to hygromycin application. In field, due to fluctuation in climatic conditions, sometimes low concentration of hygromycin is not affective (Bibi *et al*., 2013).

Histochemical GUS Reporter Assay

Histochemical analysis of GUS activity was carried out with putative hygromycin-resistant transgenic lines to confirm the transformation events. GUS expression was examined in leaves of putative transgenic plants. GUS-positive blue coloration was visibly detected in all transgenic tissues stained with X-gluc solution, whereas no blue color was observed in the non-transformed tissues (Kumar *et al*., 2010). Results of this research demonstrated that plants with blue spots were obtained in almost DNA plasmid concentration and time of stigma-drip treatments in IP3A and IP3P genotype same as result of hygromycin resistant assay, also in JcUMM18 genotipe. However, there were no significant effect of all treatments on percentage of GUS positive plants in each of three genotypes (Table 1). Percentage of GUS positive plant of IP3A, IP3P and JcUMM18 genotype ranged from 1.5-16.7%. Histochemical staining activity of glucuronidase in transgenic leaves of jatropha was shown in Figure 2. Leaves of putatively transgenic plants indicated blue color or blue spot, conversely no blue spot in non transgenic plant.

Molecular Analysis of hptII and gus Gene

To further confirm putative transgenic plants at a molecular level, PCR analysis was conducted on the *hptII* and *gus* genes that were integrated in the genomic DNA of these plant with hygromycin resistance. A DNA band was detected at a size of 500 bp and 1,000 bp in the transgenic lines by genomic PCR with primers specific for *hptII*

Figure 1. No necrotic in transgenic (A) and necrotic patches in non transgenic (B) and wild type (C) produced after application of hygromycin on leaves using leaf painting method

Table 1. Number of seedling, necrosis free plants and GUS stained plus plants of T0 generation of three jatropha genotypes in combination of concentration and application time of plasmid DNA

Treatments	Number of seedling			Necrosis free plants (%)			GUS stained + plants (%)		
	IP3A	IP3P	JcUMM18	IP3A	IP3P	JcUMM18	IP3A	IP3P	JcUMM18
Control	11.3	8.7	13.7	0.0	0.0	0.0	0.0	0.0	0.0
K1W1	9.3	11.3	14.3	4.4	6.4	0.0	4.4	6.4	0.0
K1W2	7.3	12.0	9.7	0.0	0.0	0.0	0.0	0.0	0.0
K1W3	15.0	9.0	7.0	0.0	4.4	5.6	0.0	4.4	5.6
K1W4	10.3	14.7	9.7	7.8	2.1	0.0	7.8	2.1	0.0
K1W5	15.0	11.3	9.3	3.9	6.7	0.0	3.9	6.7	0.0
K2W1	12.0	9.3	10.0	0.0	0.0	0.0	0.0	0.0	0.0
K2W2	10.7	10.3	15.3	0.0	2.1	1.7	0.0	2.1	1.7
K2W3	10.3	7.7	11.3	0.0	11.1	0.0	0.0	11.1	0.0
K2W4	15.7	13.3	13.3	0.0	0.0	0.0	0.0	0.0	0.0
K2W5	13.3	11.3	10.0	16.7	1.5	3.7	16.7	1.5	3.7
K3W1	12.3	12.7	13.7	6.7	2.4	0.0	6.7	2.4	0.0
K3W2	12.3	17.3	14.3	3.3	0.0	0.0	3.3	0.0	0.0
K3W3	10.3	12.3	9.7	5.4	0.0	0.0	5.4	0.0	0.0
K3W4	5.7	17.0	7.0	0.0	0.0	0.0	0.0	0.0	0.0
K3W5	11.3	9.3	9.7	5.6	0.0	0.0	5.6	0.0	0.0
	ns	ns	ns	ns	ns	ns	ns	ns	ns

Remarks: control = *wild type*, K1 = concentration of 0.05 µg µL^{-1}, K2 = 0.25 µg µL^{-1}, K3 = 0.50 µg µL^{-1} pCAMBIA1301, W1 = stigma-drip at 1 h, W2 = 2 h, W3 = 4 h, W4 = 7 h, W5 = 10 h after pollination, ns = non significant at α = 0.05

and *gus* respectively, while this band was not detected in non-transformed plants (Figure 3-4). The percentage of individual plants that indicated *hptII* gene in transgenic plants is less than *gus* gene in three genotypes tested. Statistically, it indicated that insertion of *hptII* and *gus* gene was not significantly different in the combination treatment

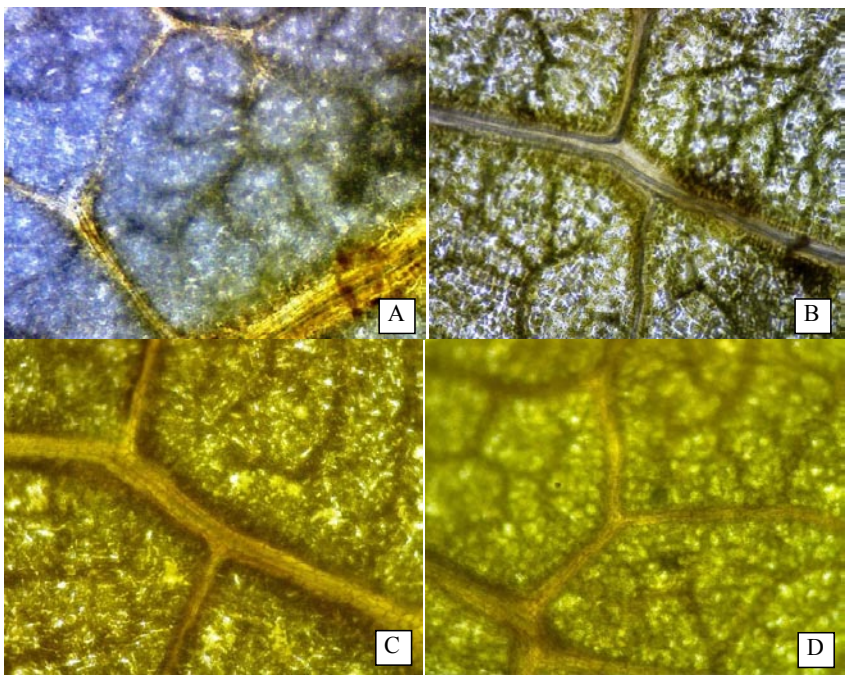

Figure 2. Histochemical staining showing activity of glucuronidase in transgenic leaves with blue spot (A & B); and no activity of glucuronidase in non transgenic (C) and wild type leaves (D)

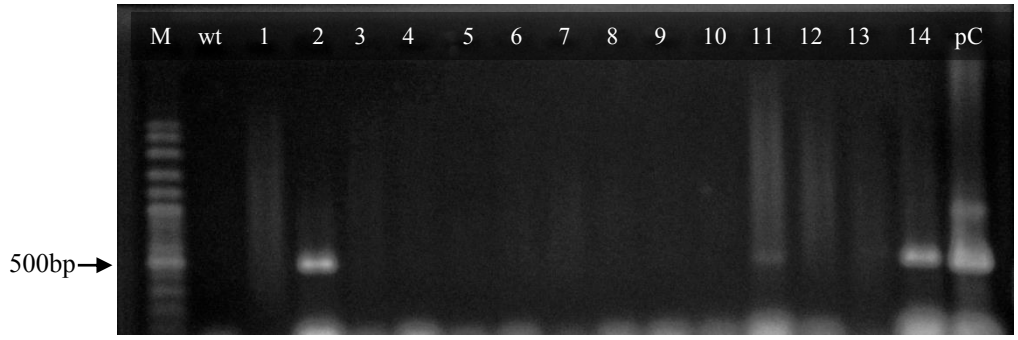

Figure 3. PCR amplification of the *hptII* gene (500 bp)
Remarks: M = marker 100bp; lanes 2, 11, 14 = putative transgenic plants; pC = pCAMBIA1301 (positive control); wt = wild type

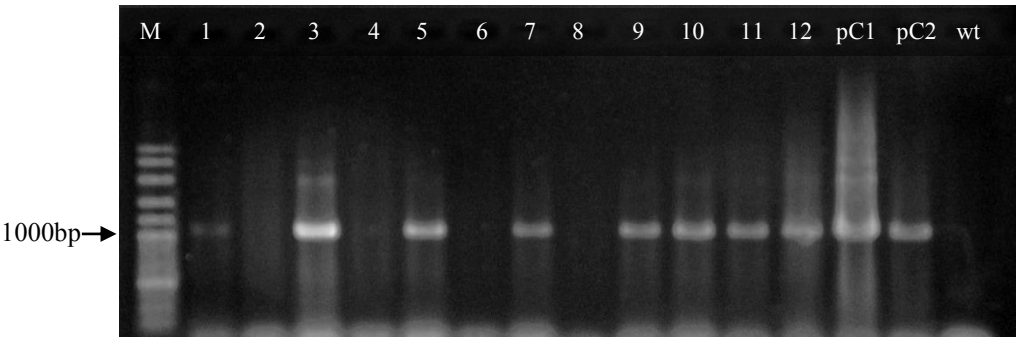

Figure 4. PCR amplification of the *gus* gene (1,000 bp)
Remarks: M = marker 100bp, lanes 1, 3, 5, 7, 9, 10-12 = putative transgenic plants. pC = pCAMBIA1301 (positive control), wt = wild type

of concentration and time application of plasmid DNA from each jatropha genotypes. The percentage of positive PCR *hptII* or *gus* gene in three genotypes of jatropha was equal to the percentage range of *hptII* and *gus* gene expression: 1.5-16.7%, but not all individuals expressing *hptII* and *gus* gene by histochemical analysis showed product positive result by PCR analysis (Table 2).

Research of jatropha transformation mediated *Agrobacterium tumefacient* conducted by Li *et al*. (2008) showed that the transformation efficiency was 13% based on the detection of b-glucuronidase activity, PCR and Southern hybridization analysis. The calculation of transformation efficiency was obtained from the 100 cotyledon explants produced 13 transgenic plants after approximately 4 months. Gu *et al*. (2014) produced one marker-free transgenic *J. curcas* line that carries a single copy of the PPepc:Cry1Ab/1Ac:TNos gene mediated by *A. tumefacient*. Joshi *et al*. (2011) reported a successful attempt of genetic transformation was made with optimized conditions using particle gene gun and establishing a stable transformation in *J. curcas* with 44.7% transformation efficiency.

Zhang *et al*. (2009) reported that the transformation efficiency of cotton transformation via pollen-tube pathway was usually relative low, ranging from 0.5% to 1%, if calculated from the setting bolls transgenically to the ovaries injected with the exogenous DNA. The low transformation efficiencies may be due to three reasons. Firstly, the DNA quality was not sufficient and the DNA concentration injected was unsuitable. The exogenous DNA without high quality usually was covalently combined with other molecules, such as proteins and polysaccharide, which could slow down the movement of the injected DNA in the pollen tube and decrease the integration rate of the exogenous DNA into the chromosome of the fertilized zygote. Secondly, high molecular weight of DNA for injection resulted in inconvenient transportation via the pollen tube, which could also affect its insertion frequency into the fertilized zygote. Thus, for the genetic transformation via pollen-tube pathway approach, the high quality and suitable length of the exogenous DNA should be considered in advance.

There were reports on cotton genetic transformation showing the transformation frequency to be dependent of genotypes (Wang *et al*., 2013). Therefore, the pollen-tube transformation pathway approach has an extra advantage of genotype independence, except for its simplicity, and easy manipulation. Putative transgenic plants of three genotipes could be gotten by pollen-tube transformation pathway.

Hao *et al*. (2011) transformed a marker-free and vector-free antisense 1-aminocyclopropane-1-carboxylic acid oxidase construct into melon via the pollen-tube pathway (the plasmid DNA solution was dripped onto the surface of the style) and obtained approximately 0.7% transformation frequency. Shan *et al*. (2012) succeeded in obtaining antisense AAT transgenic melon plants by injecting plasmid DNA into the pollinated ovary and a transformation rate of approximately 1.3% was achieved.

Tabel 2. Percentage of positive PCR *hptII* and *gus* of T0 generation of three jatropha genotypes in combination of concentration and application time of plasmid DNA

Treatments	Positive PCR *hptII* (%)			Positive PCR gus (%)		
	IP3A	IP3P	JcUMM18	IP3A	IP3P	JcUMM18
Control	0.0	0.0	0.0	0.0	0.0	0.0
K1W1	4.4	0.0	0.0	4.4	0.0	0.0
K1W2	0.0	0.0	0.0	0.0	0.0	0.0
K1W3	0.0	0.0	0.0	0.0	2.2	2.8
K1W4	0.0	0.0	0.0	4.8	2.1	0.0
K1W5	0.0	0.0	0.0	2.0	2.0	0.0
K2W1	0.0	0.0	0.0	0.0	0.0	0.0
K2W2	0.0	0.0	0.0	0.0	2.1	1.7
K2W3	0.0	11.1	0.0	0.0	11.1	0.0
K2W4	0.0	0.0	0.0	0.0	0.0	0.0
K2W5	16.7	0.0	3.7	16.7	1.5	3.7
K3W1	0.0	2.4	0.0	6.7	2.4	0.0
K3W2	3.3	0.0	0.0	0.0	0.0	0.0
K3W3	0.0	0.0	0.0	5.4	0.0	0.0
K3W4	0.0	0.0	0.0	0.0	0.0	0.0
K3W5	0.0	0.0	0.0	5.6	0.0	0.0
	ns	ns	ns	ns	ns	ns

Remarks: control=*wild type*, K1 = concentration of 0.05 µg µL^{-1}, K2 = 0.25 µg µL^{-1}, K3 = 0.50 µg µL^{-1} pCAMBIA1301, W1 = stigma-drip at 1 h, W2 = 2 h, W3 = 4 h, W4 = 7 h, W5 = 10 h after pollination, ns = non significant at α = 0.05

Chovelon *et al*. (2011) reported that 70-90% of explants expressed a transient GUS activity during the early stages of regeneration in melon transformation mediated by *A. tumefaciens mediated*, however, only 1.8-4.5% transgenic plants were obtained. These results revealed a low capacity of melon GUS-positive cells to regenerate transgenic plants. Histological analyses indicated that genetic transformation occurred in epidermal and sub-epidermal cells and reached the meristematic structures expressing a high level of GUS activity during 14 days of culture; however after this period, most of the meristematic structures showed premature cell vacuolization and disorganization. This disruption of the GUS-positive meristematic areas could be responsible of the difficulties encountered to regenerate melon and other recalcitrant plants after genetic transformation.

CONCLUSION

The efficiency of transformation via pollen-tube pathway of three jatropha genotypes ranged from 1.5-16.7% based on histochemical and molecular analysis. Sequentially by PCR analysis with specific primers *hptII* and *gus*, transgenic plants obtained in 1-3 and 3-7 of the 15 combined treatments of concentration and application time of plasmid DNA. There are indications that the transformation efficiency via the pollen-tube pathway vary in each jatropha genotypes.

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