

Research Paper

Improvement of plant regeneration and *Agrobacterium*-mediated genetic transformation of *Stylosanthes guianensis*

Mejoramiento de la regeneración de plantas y transformación genética mediada por *Agrobacterium* en *Stylosanthes guianensis*

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Abstract

As a pioneer tropical pasture legume, stylo (*Stylosanthes guianensis*) is well adapted to growth-limiting factors in acid soils. Considering the importance of stylo, there is a need to improve *Agrobacterium*-mediated genetic transformation to enable development of elite cultivars. In this study, *S. guianensis* cv. RY5 was used to systematically optimize *Agrobacterium*-mediated transformation based on its plant regeneration. Results showed that Murashige and Skoog (MS) medium containing 0.2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 2 mg/L 6-benzylaminopurine (6-BA) was the optimal callus induction medium. MS medium supplemented with 2 mg/L 6-BA was suitable for shoot regeneration from cotyledon-derived calluses, and 0.5 mg/L indole-3-acetic acid (IAA) and 0.5 mg/L indole-3-butyric acid (IBA) applications were beneficial for rooting. The highest transformation efficiency (67%) was obtained at an *Agrobacterium* concentration of optical density = 0.6 combined with an infection time of 15 min and 3 days of co-cultivation. Furthermore, 200 mg/mL carbenicillin (Carb) and 0.6 mg/L Basta[®] supplements were effective in eliminating excess bacterial growth and selecting transgenic plants, respectively. Subsequent polymerase chain reaction (PCR) analysis confirmed that the β -glucuronidase (*GUS*) and *BAR* genes were successfully integrated into the stylo genome. Wider testing of this improved protocol as a means of enhancing genetic improvement and gene function analysis of stylo seems warranted.

Keywords: Genetic engineering, GUS staining, plant hormones, tissue culture, tropical legumes.

Resumen

Como leguminosa pionera para pasturas tropicales, stylo (*Stylosanthes guianensis*) es una especie bien adaptada a los factores limitantes del crecimiento en suelos ácidos. Considerando la importancia de stylo, existe la necesidad de mejorar la transformación genética mediada por *Agrobacterium* para permitir el desarrollo de cultivares elite. En este estudio, conducido en China tropical, se utilizó *S. guianensis* cv. RY5 para optimizar en forma sistemática la transformación mediada por *Agrobacterium* con base en la regeneración de plantas. Los resultados mostraron que el medio Murashige & Skoog (MS) que contenía 0.2 mg/L de ácido 2,4-diclorofenoxiacético (2,4-D) y 2 mg/L de 6-bencilaminopurina (6-BA) fue el medio óptimo para la inducción de tejido calloso. El medio MS suplementado con 2 mg/L de 6-BA fue adecuado para la regeneración de brotes a partir de callos derivados de cotiledones, y aplicaciones de 0.5 mg/L de ácido indol-3-acético (IAA) y 0.5 mg/L de ácido indol-3-butírico (IBA) fueron beneficiosas para la formación de raíces. La mayor eficiencia de transformación (67%) se obtuvo a una concentración de *Agrobacterium* con densidad óptica de 0.6,

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combinada con un tiempo de infección de 15 min y 3 días de co-cultivo. Además, suplementos en forma de 200 mg/ml de carbenicilina (Carb) y 0.6 mg/L de Basta® fueron eficaces para eliminar el crecimiento bacteriano excesivo y seleccionar plantas transgénicas, respectivamente. El análisis posterior de PCR confirmó que los genes de la β -glucuronidasa (*GUS*) y *BAR* fueron exitosamente integrados en el genoma de stylo. Se concluye que pruebas más amplias con este protocolo mejorado, usándolo como una efectiva herramienta en el mejoramiento genético y análisis de la función de genes de *S. guianensis*, parecen justificadas.

Palabras clave: Cultivo de tejido, fitohormonas, ingeniería genética, leguminosas tropicales, tinción de GUS.

Introduction

Species of *Stylosanthes*, referred to as stylos, are important pasture legumes and widely distributed across the tropical and subtropical areas of the Americas, Africa and Asia. The genus includes more than 40 species, such as *S. guianensis* (common stylo), *S. hamata* (Caribbean stylo), *S. scabra* (shrubby stylo), *S. viscosa* (sticky stylo) and *S. seabrana* (Caatinga stylo) (Chandra 2013). Stylo is regarded as the ‘tropical alfalfa’ due to its multiple uses in tropical agricultural systems, including sown pastures, feed for livestock and improvement of soil properties, natural grassland and orchard mulching. Considering the importance of this pasture legume, it was introduced to China from Colombia and Australia in the 1960s (Tang et al. 2009). *Stylosanthes guianensis* cv. RY5, a single plant isolated from the population of its parent CIAT 184, exhibits the traits of earlier flowering, higher seed production, anthracnose resistance and cold tolerance compared with its parent, and is considered an elite stylo cultivar that is widely grown in South China (Liu et al. 2001; Tang et al. 2009).

With the development of technology, stylo has attracted large research interest in many aspects, including ecological studies (Vander Stappen et al. 1999; Sawkins et al. 2001), physiological and biochemical analyses (Zhou et al. 2005; Sun et al. 2014; Chen et al. 2015; Wang et al. 2017; Liu et al. 2018) and genetic diversity studies (Vander Stappen et al. 2000; Jiang et al. 2005; Ding et al. 2015). For example, stylo has been recognized as a pioneer tropical pasture with extensive adaptation to growth-limiting factors in acidic infertile soils, such as phosphorus (P) deficiency and aluminum (Al) and manganese (Mn) toxicity (Chen et al. 2015; Jiang et al. 2018; Liu et al. 2019). It has been demonstrated that malate synthesis in roots and exudation from roots of *S. guianensis* are the critical tolerance strategies for Al and Mn toxicity in acidic soils (Sun et al. 2014; Chen et al. 2015). Recently, a root-associated purple acid phosphatase, SgPAP23, was characterized as a primary mediator of extracellular phytate-P utilization in stylo (Liu et al. 2018).

Although much work has been conducted as described above, extensive studies of stylo are restricted by some

challenges, such as limited available germplasm resources, narrow genetic variability, lack of genome information and susceptibility to chilling stress as well as anthracnose disease (Chandra 2013; Wang et al. 2017). To solve these issues, genetic improvement through biotechnological techniques is one of the most effective and useful approaches that will help to improve the desirable traits of plant adaptation to biotic or abiotic stresses, contributing to agricultural production (Mittler and Blumwald 2010).

Over the past decades, significant development in efficient genetic transformation methods has been made in many plants. Among them, *Agrobacterium*-mediated transformation based on plant tissue culture is the most useful method for genetic transformation in various crops, such as rice (*Oryza sativa*) (Ozawa 2012), maize (*Zea mays*) (Ishida et al. 2007), sorghum (*Sorghum bicolor*) (Gurel et al. 2012), soybean (*Glycine max*) (Wang et al. 2009) and alfalfa (*Medicago sativa*) (Tesfaye et al. 2001). Although great efforts have been made to develop an *Agrobacterium*-mediated genetic transformation system for the genus *Stylosanthes*, such as *S. hamata* (Iji et al. 1995; Kumar and Chandra 2010), *S. humilis* (Manners and Way 1989), *S. seabrana* (Kumar and Chandra 2009; 2010) and *S. guianensis* (Quecini et al. 2006; Wang et al. 2008; Yuan et al. 2011; Bao et al. 2016), the genetic transformation procedure has not been adequately improved. Furthermore, combination analyses of *Agrobacterium*-mediated transformation based on plant regeneration systems are rarely performed on stylo. Only a few studies have successfully developed transgenic stylo plants thus far (Wang et al. 2008; Bao et al. 2016; Chen et al. 2016). Therefore, optimization of plant regeneration and an *Agrobacterium*-mediated transformation system are critical for the genetic improvement of stylo cultivars and basic research.

The efficiency of *Agrobacterium*-mediated genetic transformation is influenced by many factors, such as plant species and explants, the process of callus induction and differentiation, and *Agrobacterium* infection (Cheng et al. 2004; Trivellini et al. 2015). In this regard, the *S. guianensis* cultivar RY5 was used to optimize the plant regeneration system in this study, including callus

induction, shoot regeneration and root induction. Subsequently, factors influencing the *Agrobacterium*-mediated transformation system, including bacteriostatic antibiotics, *Agrobacterium* concentrations, infection and co-cultivation duration and Basta® concentrations, were analyzed according to β -glucuronidase (GUS) staining analysis. Furthermore, polymerase chain reaction (PCR) analysis was performed to examine the putative transformed stylo plants.

Materials and Methods

Plant material and explant preparation

In this study, the stylo cultivar RY5 was used. Seeds of RY5 were soaked in hot water at 80 °C for 3 min, followed by surface sterilization using 75% ethanol (v/v) and 2% sodium hypochlorite (v/v), and were subsequently germinated in Petri dishes containing basal Murashige and Skoog (MS) solid medium containing 30 g/L sucrose and 7 g/L agar (Liu et al. 2018). The pH of the medium was adjusted to 5.8 using 1 mol/L KOH or H₂SO₄. After 6–8 days germination, explants of euphylla, cotyledon and hypocotyl, approximately 10 mm in length, were used for callus induction.

Callus induction and plant regeneration

For embryogenic callus induction, the different explants were inoculated on MS solid medium containing different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) (0–0.5 mg/L) and 6-benzylaminopurine (6-BA) (0–2 mg/L) supplemented alone or in combination. All treatments had 4 biological replicates, and each replicate included 10 explants. The explants were sub-cultured every 10 days on fresh MS medium. After 30–40 days of sub-culture, a granular callus approximately 5 mm in size was recorded for callus induction.

For shoot regeneration, the embryogenic callus from the explants of the cotyledon was selected and transferred onto MS medium supplemented with different concentrations of 6-BA (0–4 mg/L) for shoot regeneration. The cultures were incubated at 28 °C under an irradiance of 80 μ mol/m/s (16 h of light:8 h of dark). All treatments had 3 biological replicates and each replicate included 10 calluses. After 8–10 weeks of culture, the shoots with 2–4 leaves that elongated from the callus were recorded.

For root induction, elongated shoots were excised from the callus and transferred onto different rooting media, including M1 (MS containing 20 g/L sucrose, 0.8 g/L activated charcoal and 7.0 g/L agar; pH 5.8), M2 [MS

containing 0.5 mg/L indole-3-acetic acid (IAA), 0.5 mg/L indole-3-butyric acid (IBA), 20 g/L sucrose, 0.8 g/L activated charcoal and 7.0 g/L agar; pH 5.8] and M3 [MS containing 0.5 mg/L IAA, 0.5 mg/L α -naphthaleneacetic acid (NAA), 20 g/L sucrose, 0.8 g/L activated charcoal and 7.0 g/L agar; pH 5.8]. After 3 weeks of culture, the rooting efficiency was determined. All experiments had 4 biological replicates and each replicate included 10 shoot buds.

Carbenicillin (Carb) concentration determination

To detect the effects of Carb on the growth of *Agrobacterium tumefaciens* strain EHA105, *A. tumefaciens* with an initial absorbance of 0.02 at 600 nm was grown in YEB liquid medium (5 g/L tryptone, 1 g/L yeast extract, 5 g/L beef extract, 5 g/L sucrose, 0.5 g/L MgSO₄·7H₂O) supplied with different concentrations (0–400 mg/mL) of Carb at 28 °C. After 48 h of growth, the absorbance of *A. tumefaciens* was recorded. To detect the effects of Carb on callus induction, cotyledon explants were incubated in the optimal callus induction MS solid medium (MS medium supplied with 0.5 mg/L 2,4-D and 2.0 mg/L 6-BA) containing different concentrations (0–1,000 mg/mL) of Carb. The numbers of embryogenic calluses were recorded after 30–40 days of culture. All experiments had 5 biological replicates and each replicate included 100 explants.

Infection and co-culture assay

The binary vector pCAMBIA3301, containing the *GUS* reporter gene driven by the cauliflower mosaic virus 35S (CaMV 35S) promoter, was used as the transformation vector in this study. The *BAR* gene was used as a selection marker under the control of the CaMV 35S promoter. *Agrobacterium tumefaciens* strain EHA105 harboring pCAMBIA3301 was grown in 50 mL of YEB liquid medium containing 50 mg/L kanamycin and 50 mg/L rifampicin at 28 °C for 48 h. After centrifugation at 5,000 rpm for 3 min, the bacterial cells were harvested and further re-suspended in YEB liquid medium.

The cotyledon explants were incubated in 50 mL of *Agrobacterium* suspension cells harboring the pCAMBIA3301 vector with different absorbances at 600 nm (from 0.2 to 1.0), which contained 50 mg/L acetosyringone (AS). The mixtures were then cultured for 5–25 min with gentle shaking. Subsequently, the *Agrobacterium*-infected explants were blotted dry on sterile filter paper and were then transferred onto co-cultivation medium (MS medium containing 0.5 mg/L 2,4-D and 2.0 mg/L 6-BA) supplied with 50 mg/L AS for

1–5 days. After that, the explants were transferred onto the callus induction MS medium (MS medium supplied with 0.5 mg/L 2,4-D and 2.0 mg/L 6-BA) containing 200 mg/mL Carb and 0.2–0.8 mg/L Basta®. After 30–40 days of culture, the transformation efficiency was determined based on GUS staining analysis as described by Qin et al. (2012). Briefly, the putative transformed callus was incubated in the GUS staining solution containing 0.2 mol/L Na₂HPO₄-NaH₂PO₄ and 1 mmol/L 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (pH 7.0) at 37 °C for 12 h, followed by washing in 70% ethanol (v/v). All experiments had 3 biological replicates and each replicate included more than 40 explants.

Putative transformed plant generation

Under the optimal procedure of infection, co-culture and selection, the basta-resistant callus was transferred onto selection medium (MS medium containing 2.0 mg/L 6-BA, 0.6 mg/L Basta® and 200 mg/mL Carb) for shoot regeneration for 8–10 weeks. Subsequently, the regenerated plantlets were transferred onto the optimal rooting medium (MS medium containing 0.5 mg/L IAA, 0.5 mg/L IBA, 20 g/L sucrose and 0.8 g/L activated charcoal; pH 5.8) for 3 weeks of root growth. All of these cultures were incubated at 28 °C under an irradiance of 80 μmol/m/s (16 h of light:8 h of dark). Finally, the putative transformed plantlets with sufficient root systems were transplanted and cultured in a 1:1 soil:sand mix for normal growth in the greenhouse.

Transgenic plant determination

For polymerase chain reaction (PCR), total genomic DNA was isolated from leaves of putative transgenic stylo plants using the cetyltrimethyl ammonium bromide (CTAB) extraction method modified by Ding et al. (2015). The primers *BARf* (5'-GGTCTGCACCATCGTCAACC-3') and *BARr* (5'-CCCACGTCATGCCAGTTCC-3') were used to amplify the *BAR* gene, and the primers *GUSf* (5'-TCGCCGATGCAGATATTCGT-3') and *GUSr* (5'-CCCGCTAGTGCCTTGTTCCA-3') were used to amplify the *GUS* gene. For semi-quantitative RT-PCR analysis, the total RNA from leaves was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. First-strand cDNA synthesis from 2 mg of DNase I-treated RNA was performed using PrimeScript reverse transcriptase (Takara, China). The RT-PCR reaction contained 1 μL of cDNA as a template. The PCR reaction was stopped after 28 cycles. The primers *SgEF-1af* (5'-GCACTGTCATTGATGCTCCC-

3') and *SgEF-1ar* (5'-TGGCACAGTTCCAATACCAC-3') were used to amplify the housekeeping *SgEF-1a* gene as an internal control. The binary vector pCambia3301 and wild-type stylo plant were used as positive and negative controls, respectively.

Statistical analysis

The callus induction rate (%) was calculated as the ratio of the numbers of induced calluses and the numbers of explants used. Shoot regeneration efficiency (%) was calculated as the ratio of the numbers of regenerated shoots and the numbers of calluses used. Rooting efficiency (%) was calculated as the ratio of the numbers of regenerated roots and the numbers of shoot buds used. The frequency of GUS-stained calluses (%) was calculated as the ratio of the numbers of GUS-stained calluses and the numbers of calluses used. All statistical analyses were performed by one-way ANOVA using the SPSS program (SPSS Institute, USA, v. 13.0).

Results

Optimization of the plant regeneration system of stylo

In this study, 3 explants, hypocotyl, cotyledon and euphylla were used to investigate the effects of different hormone supplements on embryogenic callus induction. The results (Table 1) showed that after 30–40 days of culture, callus induction rates were approximately 10% for the 3 tested explants under control treatment (without hormone addition), in which the callus displayed a pale yellow or white and watery texture. Callus induction rates were significantly increased by 2,4-D and 6-BA treatment, alone or in combination. The callus induction rates were 100, 87.5–90 and 95–100% for hypocotyl, cotyledon and euphylla, respectively, under 2,4-D treatment. Treatment with 6-BA enhanced the callus induction rates with more than 500, 500 and 380% increases in hypocotyl, cotyledon and euphylla, respectively, as compared with their respective controls. Furthermore, 2.0 mg/L 6-BA in combination with 0.2–0.5 mg/L 2,4-D supplement significantly increased the callus induction rates (approximately 100%), especially for cotyledon and euphylla, compared with their respective controls. However, no significant differences in callus induction were observed among the 3 tested explants under the same hormone treatment. Therefore, the optimal callus induction medium was MS medium containing 2 mg/L 6-BA and 0.2 mg/L 2,4-D, in which granular callus displayed a light green and compact texture (Table 1, Figures 1a–1d).

Table 1. Effects (mean ± s.e.) of different hormone treatments on callus induction in hypocotyl, cotyledon and euphylla explants of *Stylosanthes guianensis* after 30–40 days.

Hormone (mg/L)		No. of explants	Explant					
			Hypocotyl		Cotyledon		Euphylla	
2,4-D	6-BA		Mean of explants producing callus (%)	Color and texture of callus	Mean of explants producing callus (%)	Color and texture of callus	Mean of explants producing callus (%)	Color and texture of callus
0	0	40	12.5±2.5d ¹	Pale yellow/Watery	9.8±1.2c	White/Watery	10.0±1.8d	White/Watery
0	1	40	75.0±6.5c	Green/Compact	60.0±10.8b	Green tawny/Compact	47.5±7.5c	Green/Compact
0	2	40	87.5±7.5b	Dark green/Compact	60.0±9.1b	Dark green/Compact	70.0±7.1b	Dark green/Compact
0.2	0	40	100.0±0.0a	Pale yellow/Watery	87.5±7.5a	Tawny/Watery	95.0±5.0a	Tawny/Watery
0.2	1	40	100.0±0.0a	White green/Compact	95.0±5.0a	Yellow green/Compact	92.5±4.8a	Yellow green/Compact
0.2	2	40	100.0±0.0a	Light green/Compact	100.0±0.0a	Light green/Compact	100.0±0.0a	Light green/Compact
0.5	0	40	100.0±0.0a	White/Watery	90.0±4.1a	White/Watery	100.0±0.0a	White/Watery
0.5	1	40	100.0±0.0a	White green/Compact	97.5±2.5a	White green/Compact	92.5±4.8a	White green/Compact
0.5	2	40	95.0±2.9a	Green/Loosened	100.0±0.0a	Green/Loosened	100.0±0.0a	Green/Loosened

¹Values within columns followed by different letters indicate significant differences at P<0.05.

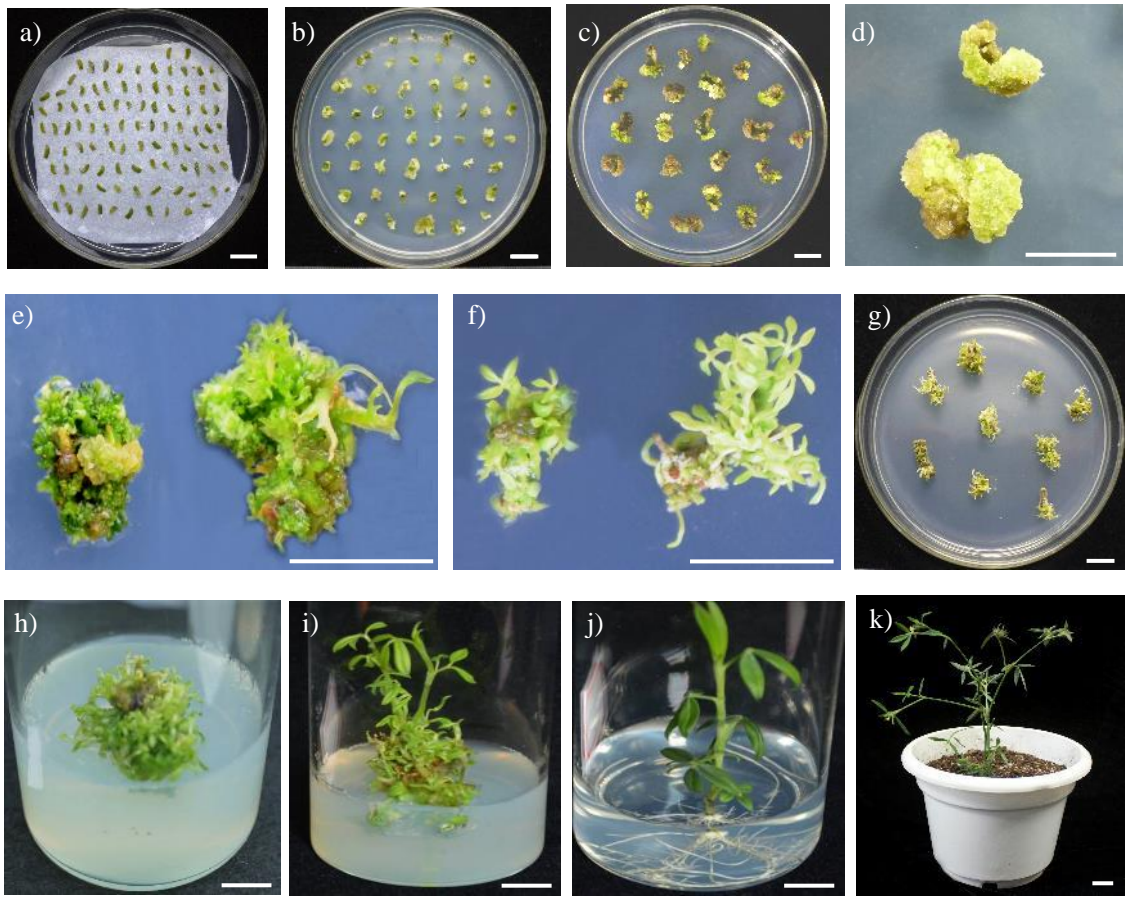


Figure 1. Plant regeneration from the cotyledon-derived callus and *Agrobacterium*-mediated transformation of *Stylosanthes guianensis*. a) Co-culture duration; b), c) and d) Callus induction; e) to i) Shoot regeneration; j) Rooting of plantlet; k) Transgenic stylo plant. Scale bar is 1 cm.

The light green and compact texture granular callus induced from the cotyledon became green and then produced a lot of tubers after cultured on the medium containing 6-BA for 50–70 days, whereas control calluses which appeared white, sticky and watery were unable to generate shoots on the differentiation medium (Figures 1e–1i). The effects of different levels of 6-BA on shoot differentiation from the cotyledon-derived callus of cv. RY5 were demonstrated as shoot regeneration rates and were significantly increased with increasing 6-BA concentrations; highest shoot regeneration rates (approximately 80.0%; $P<0.05$) were observed under 2 and 4 mg/L 6-BA supplements (Figure 2a). Subsequent rooting efficiency from the regenerated shoot after 3 weeks of root induction was higher in M2 and M3 media with different hormone supplements than in M1 medium without

hormone addition (Figure 2b). The highest rooting efficiency (100%) was observed in M2 medium containing 0.5 mg/L IAA and 0.5 mg/L IBA (Figure 1j, Figure 2b).

Improvement of *Agrobacterium*-mediated transformation

Growth of *Agrobacterium* was significantly inhibited by increasing Carb concentrations compared with the control without Carb addition (Figure 3a) with decreases of 69.6 and 98.8% under 50 and 200 mg/mL Carb treatments, respectively, compared with the control. Callus induction rate was significantly ($P<0.05$) decreased when Carb concentrations were higher than 600 mg/mL with decreases of 29.2 and 66.6% under 800 and 1,000 mg/mL Carb treatments, respectively, compared with the control (Figure 3b).

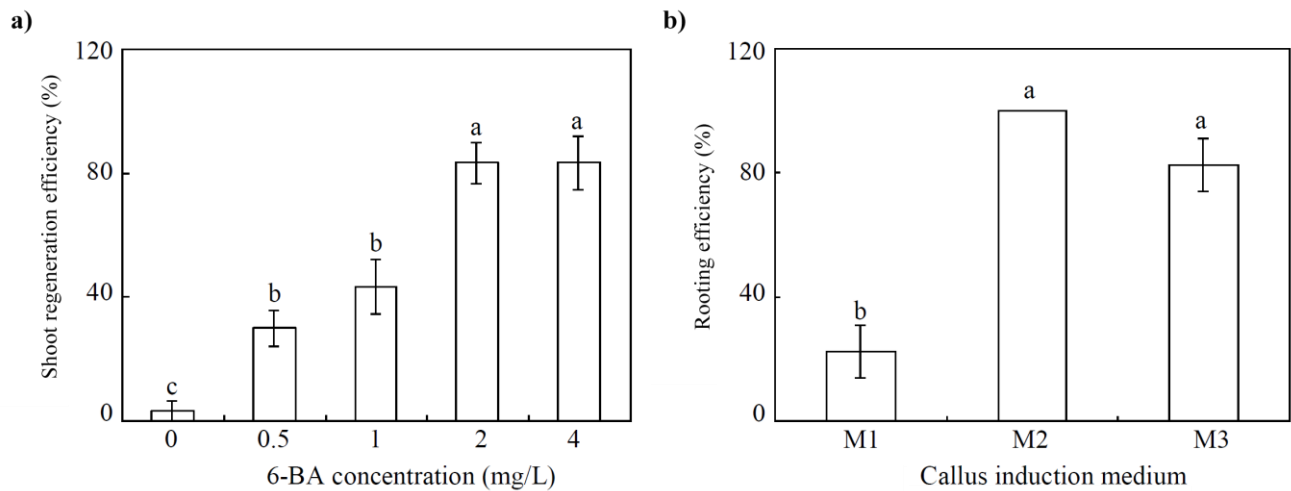


Figure 2. Effects of different hormone treatments on: a) Shoot regeneration efficiency; and b) Rooting efficiency of *Stylosanthes guianensis*. Vertical bars indicate the standard errors of means. Different letters on columns indicate significant differences at $P<0.05$.

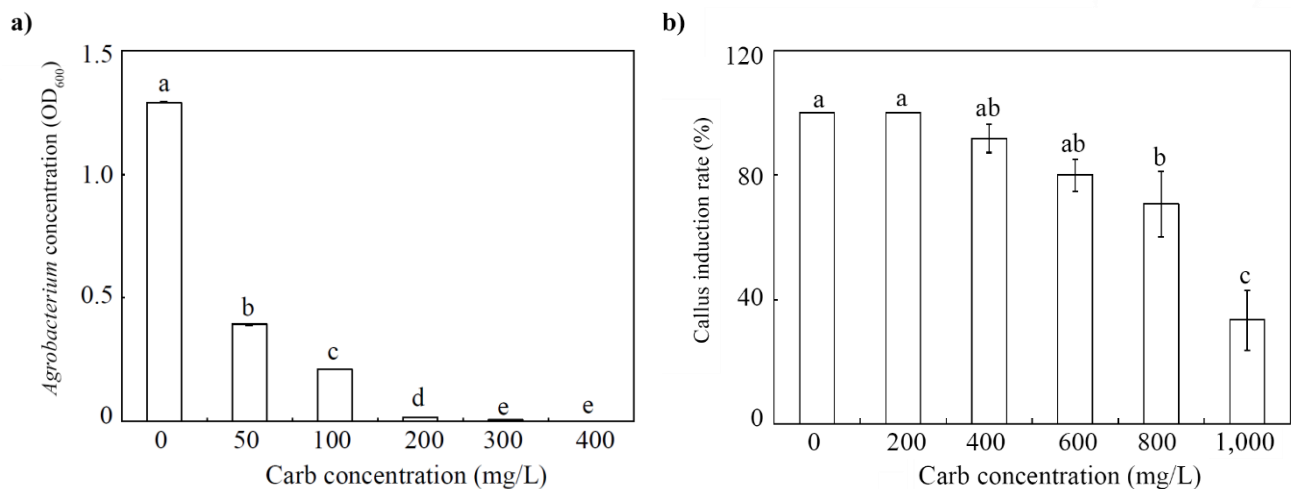


Figure 3. Effects of Carb on: a) *Agrobacterium* concentration; and b) Callus induction rate of *Stylosanthes guianensis*. Vertical bars indicate the standard error of the mean. Different letters on columns indicate significant differences at $P<0.05$.

Subsequently, the effects of *Agrobacterium* concentration, infection time and co-cultivation duration on callus transformation efficiency were evaluated based on GUS staining analysis (Table 2). A callus with blue coloration was considered a positive transformed callus after GUS staining. Highest transformation efficiency (67.5%) was observed at an *Agrobacterium* concentration of optical density (OD₆₀₀) of 0.6 and transformation efficiency decreased when the *Agrobacterium* concentration (OD₆₀₀) exceeded 0.6. Similarly, transformation efficiency increased with increasing *Agrobacterium* infection time from 5 to 20 min, and the highest transformation efficiency (59.3%; $P < 0.05$) was achieved with infection times of 15 and 20 min. However, transformation efficiency was decreased significantly (by 93.8%) after 25 min of infection compared with that after 15 min of infection. The transformation efficiency was also affected by different co-cultivation durations. The maximum transformation efficiency was obtained under 3 days of co-cultivation, when it reached 69.4%.

Additionally, the effects of Basta® on transformed callus selection were also examined and showed that transformation efficiencies varied among different Basta® treatments (Table 2). Transformation efficiency increased with increasing Basta® concentrations, peaking at 0.6

mg/L at a value of 62.1%, but then decreased at Basta® concentrations greater than 0.6 mg/L. Therefore, 0.6 mg/L Basta® was chosen for appropriate callus selection.

Transformed plant production

Subsequently, cotyledon explants were used for transformation under the optimized procedure of an *Agrobacterium* concentration (OD₆₀₀) of 0.6 with 15 min of *Agrobacterium* infection and 3 days of co-cultivation (Figure 4). The infected explants were transferred onto MS medium containing 0.2 mg/L 2,4-D, 2.0 mg/L 6-BA and 200 mg/L Carb for 1 week of recovery growth. After that, the explants were transferred onto callus induction medium containing 0.2 mg/L 2,4-D, 2.0 mg/L 6-BA, 0.6 mg/L Basta® and 200 mg/mL Carb for 30–40 days. A mass of basta-resistant calluses was generated and then transferred onto shoot regeneration medium containing 2.0 mg/L 6-BA, 0.6 mg/L Basta® and 200 mg/mL Carb for 8–10 weeks. The regenerated plantlets were cut and further transferred onto rooting medium containing 0.5 mg/L IAA and 0.5 mg/L IBA for 3 weeks. Finally, the putative transformed plantlets with a sufficiently vigorous root system were transplanted and cultured into a soil and sand mixture (1:1) in a greenhouse for normal growth (Figure 4, Figure 1k).

Table 2. Different factors affecting the transformation efficiency of *Stylosanthes guianensis*.

Factor		No. of explants	No. of resistant calluses	GUS-stained rates (%) ¹
<i>Agrobacterium</i> concentration (OD ₆₀₀)	0.2	139	138	53.2±4.60ab ²
	0.4	121	120	52.5±2.50ab
	0.6	136	134	67.5±8.26a
	0.8	122	122	36.1±1.95b
	1	153	150	38.5±8.74b
Infection time (min)	5	141	139	28.4±6.03b
	10	141	140	31.6±7.20b
	15	159	157	59.3±9.44a
	20	129	125	56.6±6.30a
	25	135	133	3.65±1.30c
Co-cultivation time (days)	1	121	120	29.2±5.07c
	2	125	121	32.3±4.51bc
	3	133	130	69.4±3.78a
	4	140	137	45.2±2.32b
	5	129	128	32.3±5.35bc
Basta® concentration (mg/L)	0.2	122	120	24.2±2.20c
	0.4	129	125	49.4±3.78b
	0.6	130	126	62.1±3.08a
	0.8	120	120	46.7±5.46b

¹GUS-stained rate calculated from the ratio of the numbers of GUS-stained calluses and the numbers of resistant calluses for each treatment. Data are mean values of 3 replications with standard error. ²Values within factors followed by different letters indicate significant differences at $P < 0.05$.

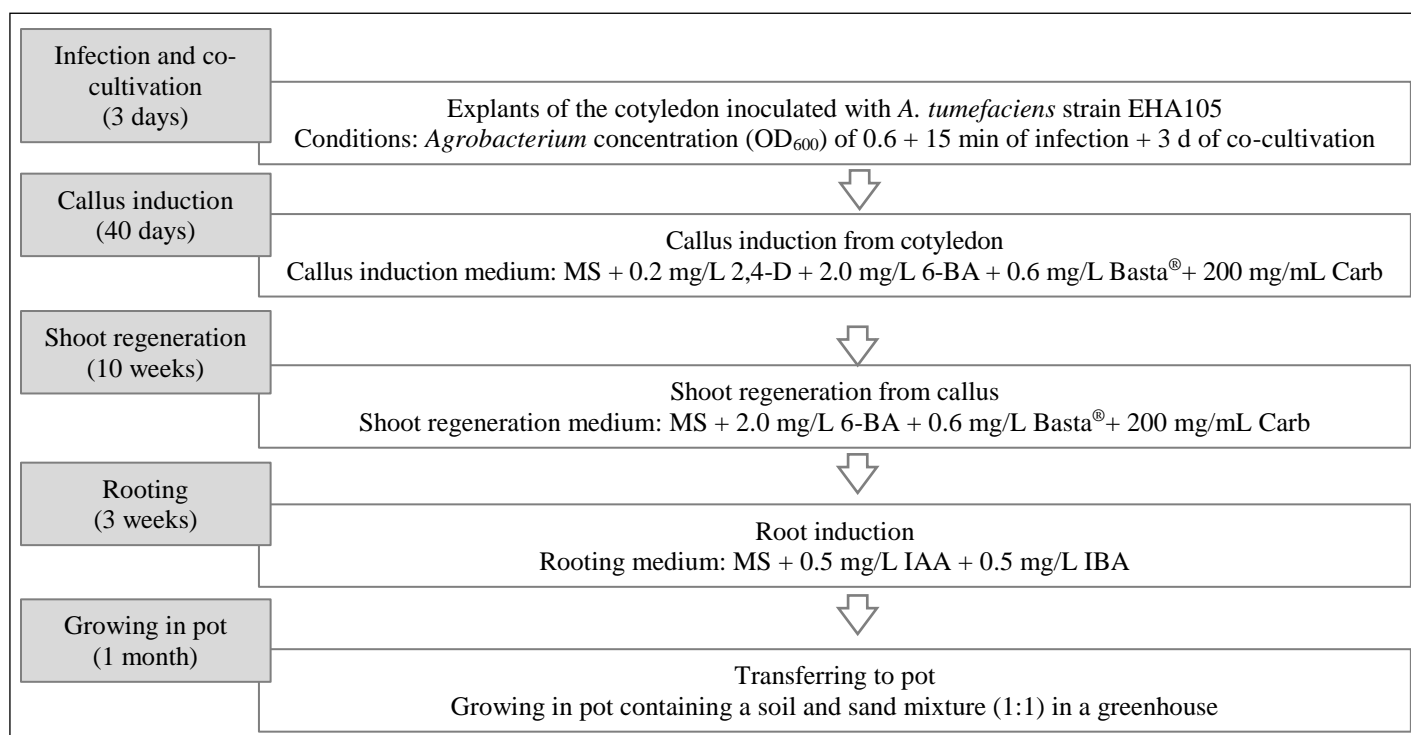


Figure 4. Optimal protocol for *Agrobacterium*-mediated transformation of *Stylosanthes guianensis*.

Determination of putative transgenic stylo plants

As shown in Figure 5, clear GUS staining was observed in callus, leaf, root and stem of transgenic plants, suggesting that *GUS* gene was successfully expressed in different tissues of stylo plants. In this study, a total of 850 explants were used for *Agrobacterium*-mediated transformation according to the optimized procedure, and 51 putative

transformed lines were obtained. To further verify the integration of foreign genes into the stylo genome, genomic DNA extracted from leaves of 15 putative transformed lines was used for PCR analysis. The results showed that 426 and 533 bp of the expected DNA fragments of the respective *BAR* and *GUS* genes were successfully amplified from 9 out of 15 randomly selected lines (Figures 6a–6c), suggesting the successful integration of foreign

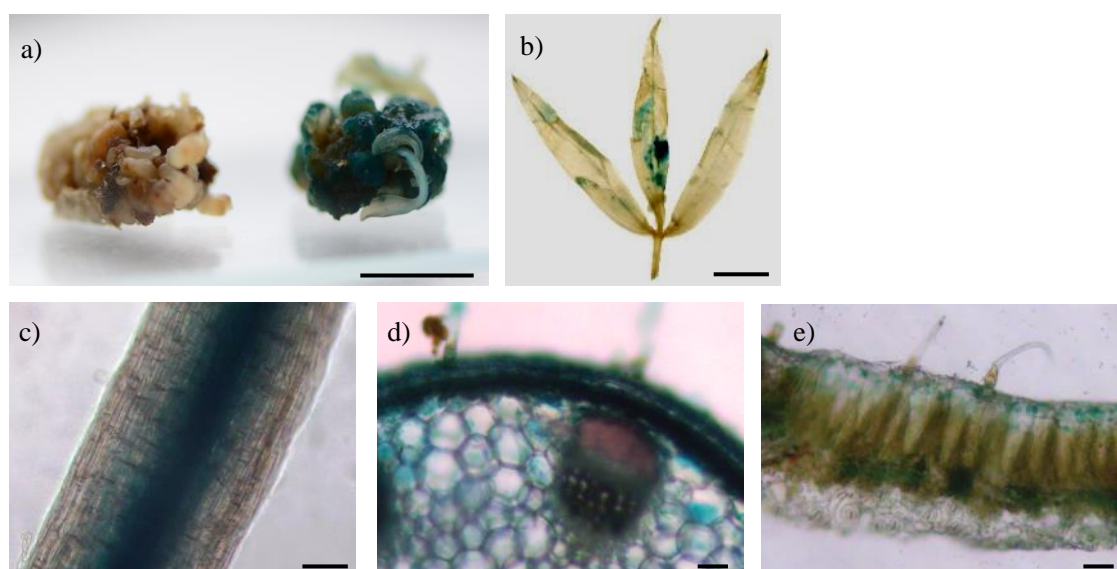


Figure 5. GUS staining of putative transformed *Stylosanthes guianensis* tissues: a) and b) GUS staining of callus and leaf, respectively; c) GUS staining of mature root; d) and e) Cross-section of stem and leaf. Scale bar in a) and b) is 1 cm. Scale bar in c), d) and e) is 100 μ m.

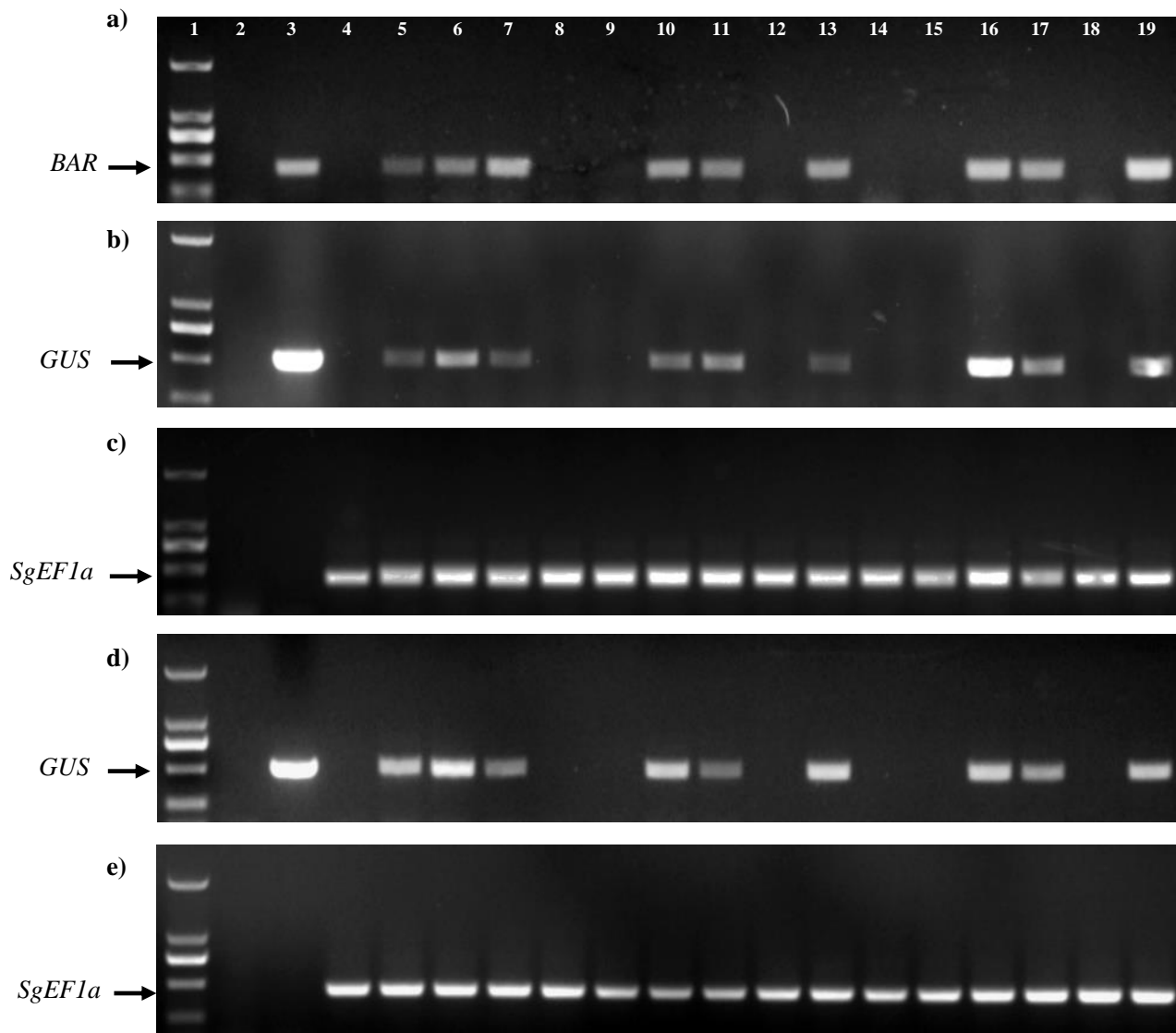


Figure 6. Molecular analysis of the putative transgenic *Stylosanthes guianensis* plants: a) Genomic PCR detection of the *BAR* gene; b) Genomic PCR detection of the *GUS* gene; c) Genomic PCR detection of the housekeeping *SgEF1a* gene; d) Expression of the *GUS* gene via RT-PCR analysis; and e) RT-PCR analysis of the *SgEF1a* gene. 1 = DL2000 DNA marker; 2 = negative control; 3 = positive control; 4 = wild type plant; and 5–19 = 15 putative transgenic lines.

genes into the stylo genome. Furthermore, the expression of the *GUS* gene in the leaves of the 15 tested putative transgenic stylo lines was also detected using RT-PCR analysis. The results showed that the foreign *GUS* gene was stably expressed in 9 out of 15 tested transgenic stylo lines (Figures 6d and 6e). In total, 33 out of 51 putative lines were proved to be positive transgenic lines, resulting in a transformation efficiency of 3.88%.

Discussion

The *Agrobacterium*-mediated genetic transformation approach is widely used for the development of transgenic

plants (Cheng et al. 2004). Despite the importance of the *Stylosanthes* genus for pasture, only a few transgenic plants have been generated and used for gene function analysis of *Stylosanthes* species (Wang et al. 2008; Bao et al. 2016), suggesting that the existing protocols might not be efficient for the development of transgenic stylo plants. Therefore, it is essential to improve the *Agrobacterium*-mediated genetic transformation procedure for stylo based on its plant regeneration.

This study has shown that MS medium containing 0.2 mg/L 2,4-D and 2 mg/L 6-BA was the optimal callus induction medium, especially for cotyledon and euphylla explants (Table 1). As growth regulators, 2,4-D, 6-BA

and NAA have been generally used for callus induction of stylo ([Wang et al. 2008](#); [Yuan et al. 2011](#); [Bao et al. 2016](#)). Yuan et al. (2011) found that the optimal condition for callus induction from cotyledons of *S. guianensis* cv. Reyan 2 was MS medium containing 3.0 mg/L 2,4-D with highest rates of callus induction reaching 74%, which is lower than that measured in this study. On the other hand, Godwin et al. (1987) showed the optimal callus induction medium for *S. scabra* leaves was MS medium supplemented with both 2,4-D and 6-BAP. These variations in appropriate hormone application can be attributed to different stylo species, cultivars or genotypes. Similar variations have also been observed in other plant species. For instance, a combination of 0.5 mg/L 2,4-D and 2.0 mg/L 6-BA resulted in better callus induction from *Vitis vinifera* flowers ([Dai et al. 2015](#)), while 2.0–3.0 mg/L 2,4-D was the optimal concentration for promoting callus induction in leaves of *Urochloa* ([Yaguinuma et al. 2018](#)).

In addition to callus induction, the differentiation of shoots from the callus is also essential for plant regeneration. Shoot organogenesis induced from the callus can be improved by application of appropriate hormone ([Cheng et al. 2004](#); [Yaguinuma et al. 2018](#)). In this study, although shoot differentiation from the cotyledon-derived callus of cv. RY5 was observed in hormone-free MS medium, the percentage of shoot regeneration was only 3.3% (Figure 2a). Our findings that MS medium with 2 mg/L 6-BA applications was most efficient for shoot regeneration (Figure 2a), producing a shoot regeneration rate of approximately 80%, is consistent with earlier findings that 2–4 mg/L 6-BA applications significantly increased the regeneration of shoots from the cotyledon-derived callus of cv. Reyan 2 ([Yuan et al. 2011](#)). Similarly, a high frequency of shoot regeneration was observed from the leaf-derived callus of *S. scabra* on MS medium containing 2.0 mg/L BAP ([Godwin et al. 1987](#)) and from the cotyledon-derived callus of *S. macrocephala* on MS medium supplemented with 0.1 mg/L NAA and 0.4 mg/L BAP ([Vieira et al. 1990](#)). However, other factors, such as callus derived from different types of explants and callus states (e.g. color and texture) at different hormone treatments, may influence the regeneration capacity of shoots in stylo, which merits further study.

The increases in rooting efficiency we observed with hormone applications support the increased rooting efficiencies of *S. seabrana* and *S. hamata* in response to the application of IAA and IBA alone or in combination compared with untreated controls ([Kumar and Chandra 2010](#)). MS medium containing NAA and IBA is beneficial for rooting of *S. guianensis* ([Kelemu et al.](#)

[2005](#)). In contrast, it has been reported that hormone-free medium is suitable for rooting of *S. scabra* and *S. guianensis* ([Valarini et al. 1997](#); [Quecini et al. 2006](#); [Bao et al. 2016](#)). We found that the appropriate rooting medium was MS medium supplemented with 0.5 mg/L IAA and 0.5 mg/L IBA, in which rooting efficiency was significantly higher than that of hormone-free MS medium (Figure 2b). Similar results have been found for other plants, such as white ash (*Fraxinus americana*) and grass pea (*Lathyrus sativus*), where IAA and IBA applications promoted rooting efficiency ([Barpete et al. 2014](#); [Palla and Pijut 2015](#)). Taken together, the results indicate that stable plantlet regeneration was optimized in cv. RY5 using cotyledons as explants.

Carb is used for terminating rudimentary *Agrobacterium* growth at suitable doses after the co-culture period during T-DNA insertion into the plant chromosome ([Li et al. 2015](#)). In our study, a dose-response experiment showed that 200 mg/mL Carb efficiently inhibited excess bacterial growth but did not affect callus induction. Optimal Carb concentrations that inhibit excess *Agrobacterium* growth range from 200 to 1,000 mg/L (Figure 3). For example, it has been demonstrated that 200, 500 and 1,000 mg/L Carb are efficient for the elimination of *Agrobacterium* in grapes (*Vitis vinifera*) ([Dai et al. 2015](#)), loblolly pine (*Pinus taeda*) ([Tang et al. 2004](#)) and tobacco (*Nicotiana tabacum*) ([Nauerby et al. 1997](#)), respectively, during bacterial infection.

Agrobacterium concentration, infection and co-cultivation duration are important factors that affect transformation efficiency. An inappropriate concentration of *Agrobacterium* may produce toxic contamination events or may not be effective for the callus, and a long or short infection time may result in a low frequency of transformation ([Cheng et al. 2004](#); [Sharma et al. 2011](#); [Trivellini et al. 2015](#)). In this study, the highest transformation efficiency (67%) was obtained under the conditions of an *Agrobacterium* concentration of $OD_{600} = 0.6$, combined with an *Agrobacterium* infection time of 15 min and 3 days of co-cultivation based on GUS staining analysis (Table 2). This finding is similar to the *Agrobacterium* concentration of $OD_{600} = 0.4$ – 0.6 , an infection time of 10 min and a co-cultivation duration of 2–3 days found to be optimal conditions for transformation of baby bamboo (*Pogonatherum paniceum*) ([Li et al. 2015](#)) and white ash (*Fraxinus americana*) ([Palla and Pijut 2015](#)). Furthermore, *Agrobacterium* infection for 2–10 min with 2–4 days of co-cultivation has been shown beneficial for increasing transformation efficiency in other stylo species, e.g. *S. humilis* and *S. guianensis* ([Manners 1987](#); [Manners and Way 1989](#); [Bao et al. 2016](#); [Chen et al. 2016](#)).

Basta® is commonly used for the selection of transgenic plants that contain the herbicide-resistant selection marker *BAR* gene, which is resistant to Basta® (Lin et al. 2009; Mayavan et al. 2015). Optimization of the Basta® concentration for selection pressure is important for increasing transformation efficiency. In this study, 0.6 mg/L Basta® was found to be suitable for transformed callus selection (Table 2), which is similar to the level recommended by Bao et al. (2016). It is noteworthy that *Agrobacterium*-mediated genetic transformation efficiency is influenced by many factors, such as phytohormone, antibiotic, growth medium and bacterial infection process (Nandakumar et al. 2004; Sharma et al. 2011). Therefore, in this study, the transformation efficiency of stylo may have been potentially affected by different hormone supplements and culture media in the regeneration and rooting processes, which need to be studied further.

The total of 33 positive transgenic plants we generated from 850 explants of the cotyledon, representing a transformation efficiency of 3.88%, was higher than that observed in *S. humilis* cv. Paterson, where only 0.3% of explants generated transgenic plants (Manners 1988). However, it is similar to the transformation efficiency of 3.47% in *S. guianensis* cv. Mineirão using microparticle bombardment reported by Quecini et al. (2006). Besides, only 25% of the putative transformed *S. guianensis* cv. RY5 lines generated from hypocotyl-derived calluses by Chen et al. (2016) proved to be positive transgenic plants, which is lower than the frequency of 64.7% obtained from cotyledons herein (Figure 6). It appears that the transformation efficiency of stylo was improved using the optimal protocol in the current study.

In conclusion, this study has developed an optimal strategy for the *Agrobacterium*-mediated genetic transformation of cv. RY5 based on its plant regeneration system. This optimal procedure provides an appropriate platform to investigate the molecular mechanisms underlying stylo adaptation to environmental stresses. Furthermore, based on the optimized protocol, we can modify the specific traits of various stylo varieties by biotechnological and molecular approaches, which are beneficial for the genetic improvement of stylo.

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