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Effect of inoculation time on *Agrobacterium*-mediated transformation efficiency of *Musa* cv. 'Grande naine' (AAA)

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ABSTRACT
Increasing inoculation time during *Agrobacterium*-mediated transformation has been shown to favour transformation efficiency in several plant species. The effect of inoculation time in combination with spermidine (Spd) on efficiency was determined during *Agrobaterium*-mediated transformation of the *Musa* cultivar 'Grande naine' (AAA). Banana embryogenic cell suspensions (ECSs) were incubated with the bacterial strain EHA 105 carrying the binary vector pFAJ3000. Six different inoculation conditions (6 h, 6 h+Spd, 12 h, 12 h+Spd, 24 h, 24 h+Spd) were compared based on transient GUS expression and number of embryo colonies formed. Moreover, leaf fragments from 24 randomly chosen regenerated plantlets were assayed presence and expression of the transgenes. Consequently, samples that were inoculated for 24 h in medium supplemented with 1 mM spermidine showed the highest transformation efficiency, expressed as number of blue foci and regenerated colonies after the selection treatment. Here we showed for the first time that longer inoculation times in combination with spermidine enhance the efficiency of *Agrobacterium*-mediated transformation of the banana cultivar 'Grande naine'.

Keywords: banana, β-glucuronidase, genotype, somatic embryo, PCR

Efecto del tiempo de inoculación en la eficiencia de la transformación mediada por *Agrobacterium* de *Musa* cv. 'Grande naine' (AAA)

RESUMEN
El incremento en el tiempo de inoculación durante la transformación mediada por *Agrobacterium* ha mostrado que aumenta la eficiencia de la transformación en varias especies de plantas. En este trabajo se determinó el efecto del tiempo de inoculación en combinación con la adición de espermidina (Spd) en la eficiencia de la transformación genética mediada por *Agrobacterium tumefaciens* del cv. 'Grande naine' (*Musa* AAA). Las suspensiones celulares embriogénicas de banano fueron inoculadas con la cepa bacteriana EHA 105 que contiene el vector binario pFAJ3000. Se compararon seis condiciones de inoculación (6 h, 6 h+Spd, 12 h, 12 h+Spd, 24 h, 24 h+Spd) en cuanto a la expresión transitoria GUS y el número de colonias embriogénicas formadas. Además, se analizaron fragmentos de hojas de 24 plántulas regeneradas para la presencia y expresión de los transgenes. Consecuentemente, las muestras por 24 h con 1 mM de espermidina mostraron la mayor eficiencia de transformación, expresada en número de puntos azules y colonias regeneradas después de la selección. En este trabajo se muestra por primera vez que el aumento del tiempo de inoculación en combinación con el uso de espermidina incrementa la eficiencia de la transformación genética mediada por *Agrobacterium* en el cultivar 'Grande naine' de banano.

Palabras clave: banano, β-glucuronidasa, genotipo, suspensiones celulares embriogénicas, PCR

INTRODUCTION

Bananas and plantains (*Musa* spp.) are staple food for over 400 million people worldwide, serving as a rich source of carbohydrates, vitamins and mineral elements like potassium, phosphorous, calcium and magnesium (Mohapatra *et al*., 2010).

The banana cv. 'Grande naine' (*Musa* AAA) is one of the most commercialized cultivar

worldwide due to its resistance to wind throw and production of large bunches and fingers despite its relatively small stature (Ploetz *et al*., 2007). This cultivar shows enhanced resistance against races 1, 2 and 3 of the devastating pathogen *Fusarium oxysporum* L. f. sp. *cubense* (Foc), whereas it is highly susceptible to the Black Leaf Streak Disease caused by *Mycosphaerella fijiensis* Morelet and the emerging tropical race 4 of Foc (Robinson and Galán, 2010). Due to polyploidy, long life cycle and high sterility found in this and most of edible cultivars, genetic transformation arises as an attractive means for introducing agronomically important traits and performing functional genomics analysis in *Musa* species (Roux *et al*., 2008). Therefore, the development of protocols that render high transformation efficiencies and could be used for several cultivars is essential (Khanna *et al*., 2004).

Since Ságі *et al*. (1994) first reported the genetic transformation in this genus through protoplast electroporation, several methods have been developed. The *Agrobacterium tumefaciens*-mediated transformation of ECSs is the most commonly used (Ganapathi *et al*., 2001; Khanna *et al*., 2004; Pérez-Hernández *et al*., 2006 a; Ghosh *et al*., 2009; Chong-Pérez *et al*., 2012 a). Likewise, several parameters have been modified in order to increase the transformation efficiency. Recently, Chong-Pérez *et al*. (2012 a) reported the use of spermidine (Spd) and modifications on inoculation time (30 min and 6 h) in a centrifugation-assisted transformation protocol to increase transformation efficiency of banana cv. 'Dwarf Cavendish' (*Musa* AAA), with the best results for 6 h of inoculation in the presence of spermidine.

Increasing inoculation time during *Agrobacterium*-mediated transformation has been shown to favour transformation efficiency in several plant species (Dan and Ow, 2011). However, the use of longer inoculation times is limited by the induction of defense responses in the plant tissues, which typically involve necrosis or programmed cell death (Hansen, 2000; Zhang *et al*., 2013). Thus, the regeneration of explants and consequently the transformation efficiency are compromised. Spermidine is a major polyamine in plants that has been used in genetic transformation since it favours embryo multiplication and regeneration (Petri *et al*., 2005; Silva *et al*., 2009) and enhance T-DNA transfer and activation of *vir* genes when added exogenously (Kumar and Rajam, 2005).

Since genetic transformation in *Musa* species is far for being a stablished routine, mostly due to transformation dependency on genotype, the main objective of this work was to determine whether increasing the inoculation time of embryogenic cell aggregates with *A. tumefaciens* in the presence of spermidine influence transformation efficiency in *Musa* cv. 'Grande naine'.

MATERIALS AND METHODS

Inoculum preparation

The *A. tumefaciens* strain EHA-105 carrying the binary vector pFAJ3000 (De Bondt *et al*., 1994) was used in this study (Figure 1). The bacteria were grown on semi-solid LB medium supplemented with spectinomycin 100 mg l^{-1}, streptomycin 300 mg l^{-1} and rifampicin 50 mg l^{-1}, and incubated at 28 °C for 24–48 h. Resulting single colonies were cultured in 3 ml selective liquid LB medium and incubated at 200 rpm and 28±2 °C for 16–24 h to an A_{600} of approximately 1.2 units (Biophotometer, Eppendorf, Germany). Then, 50 ml selective liquid YEP medium were inoculated with 50 µl of this culture and kept under the preceding conditions to an A_{600} of 1.2 units. After that, the culture was centrifuged for 10 min at 3 430 x g (Eppendorf, Germany) and the cells were re-suspended in liquid ZZ medium (half strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), MS vitamins, 10 mg l^{-1} ascorbic acid, 30 g l^{-1} sucrose, 1 mg l^{-1} 2,4-D, pH 6.12 before autoclaving) supplemented with 200 µM acetosyringone. The A_{600} was adjusted between 0.4 and 0.5 units (~10^9 cfu ml^{-1}).

Plant material

Embryogenic cell suspensions (ECSs) from *Musa* cv. 'Grande naine' (AAA) were obtained from immature male flowers as described by Côte *et al*. (1996).

Genetic transformation and plant regeneration

Genetic transformation of cell aggregates and plant regeneration were performed according

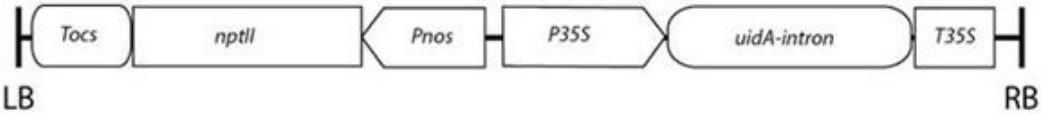

Figure 1. Schematic representation of the T-DNA region of the binary vector pFAJ3000. LB and RB, left and right border respectively; *P35S* and *T35S,* cauliflower mosaic virus 35S gene promoter and terminator, respectively; *uidA*-intron, intron-interrupted *β-glucuronidase* gene; *Pnos*, nopaline synthase gene promoter; *nptII,* neomycin phosphotransferase gene; *Tocs*, octopine synthase polyadenylation signal.

to Chong-Pérez *et al*. (2012 b). Two hundred microliter of 33% settled cell volume (SCV) ECSs in ZZ liquid medium were mixed with one milliliter of bacterial suspension in 24-well plates and incubated at 25°C and 25 rpm in the dark. During inoculation with the bacteria, five different conditions (6 h, 12 h, 12 h+Spd, 24 h, 24 h+Spd) were compared to the standard condition(6 h with 1 mM Spd) (Chong-Pérez *et al*., 2012 a). After that, cells were transferred to a nylon mesh, placed in Petri dishes containing ZZ medium, 3 g l^{-1} Gelrite and 200 µM acetosyringone, pH 6.3 before autoclaving (final pH after autoclaving ~5.6), and kept in the dark at 21°C for six days. Then, cell aggregates were shifted to semi-solid ZZ medium, pH 6.12 before autoclaving, containing 200 mg l^{-1} timentin (to eliminate the bacteria) and 50 mg l^{-1} geneticin (to select for transformed cells), and kept in the dark at 27 ± 2°C for eight weeks, with bi-weekly subcultures. Next, the obtained embryo colonies (groups of proembryos and embryos derived from a single embryo, and consequently from the same transformation event) were placed in antibiotic containing RD1 medium (half strength MS medium, MS vitamins, 100 mg l^{-1} ascorbic acid, 100 mg l^{-1} myo-inositol, 2.5 g l^{-1} Gelrite, 30 g l^{-1} sucrose, pH 5.8) for four weeks in the dark at 27 ± 2°C. Resulting embryos were transferred to maturation medium (MS medium, MS vitamins, 0.25 mg l^{-1} 6-bencylaminopurine (6-BAP), 0.75 mg l^{-1} indole-3-acetic acid (IAA), 100 mg l^{-1} myo-inositol, 45 g l^{-1} sucrose, 2.5 g l^{-1} Gelrite, pH 5.8) for another three weeks. Afterwards, mature embryos were placed in germination medium (MS medium, 1.0 mg l^{-1} biotin, 0.5 mg l^{-1} 6-BAP, 2.0 mg l^{-1} IAA, 45 g l^{-1} sucrose, 2.5 g l^{-1} Gelrite, pH 5.8) at 27 ± 2°C under solar light conditions. After four weeks in this conditions, regenerated plants were transferred to elongation medium (MS medium, 30 g l^{-1} sucrose, 2.5 g l^{-1} Gelrite, pH 5.8) for another month.

Histochemical β-glucuronidase (GUS) assays

Histochemical GUS assay was conducted as described before (Jefferson *et al*., 1987; Chong-Pérez *et al*., 2012 a) on embryogenic cell aggregates and leaf segments. Transient GUS expression (TGE), expressed as the average number of blue foci of 30 samples per treatment (200 µl at 33% settled cell volume of ECSs ~50 mg fresh weight) was determined. In order to determine genetic transformation efficiency, the number of blue foci on embryogenic cells and embryo colonies were determined after the co-culture and the eight weeks selection period, respectively. Likewise, 1 cm^2 leaf segments sliced from 24 randomly chosen regenerated plantlets (four plantlets per treatment) were incubated in the assay solution supplemented with 0.2% SDS and 1% (w/v) ascorbic acid and subjected to 66.6 kPa vacuum for 10 min to favor substrate penetration into the tissues. Incubation was performed as above and the tissues were washed in 70% (v/v) ethanol to improve color contrast. Consequently, β-glucuronidase stable expression was qualitatively assessed through the presence of blue zones.

DNA isolation and PCR analysis

The above 24 plantlets were also analysed by PCR. The genomic DNA was isolated according to the protocol of Khayat *et al*. (2004) with modifications described by Chong-Pérez *et al*. (2012 b). The sequence of the primers used to detect the presence of the *uidA-intron* and *nptII* genes were Fw 5'-TGGGCAGGCCAGCGTATCGT-3', Rv5'-ATCACGCAGTTCAACGCTGAC-3', and Fw 5´-ATGATTGAACAAGATGGATTGCACGC-3´, Rv 5´-TGATGCTCTTCGTCCAGATCATC-3´, respectively. The expected fragments from the amplification reactions were 609 bp and 488 bp length, respectively. The reaction was performed in 25 µl final volume mixture containing 0.4 µg of genomic DNA, 0.5 µM of

each primer, 200 µM dNTPs, 1X DreamTaq buffer, 1 unit DreamTaq polymerase (Fermentas, Germany), 1% BSA and 1% (w/v) PVP. Thermocycling was carried out in a Mastercycler programmable thermal control (Eppendorf, Germany) and started with denaturation at 94 °C for 4 min, followed by 30 cycles at 94 °C for 45 s, 66 °C for 45 s and 72 °C for 45 s, with a final extension at 72 °C for 10 min. The amplified fragments were analysed by 1.5% (w/v) agarose gel electrophoresis performed at 10 V/cm in 1X Tris-borate-EDTA buffer and stained with 0.5 µg ml^{-1} ethidium bromide.

Statistical analysis

Data were processed using the IBM Statistical Package for Social Sciences (SPSS) for Windows Version 22.0 and non-parametric tests H of Kruskal Wallis and U of Mann Whitney were used with a significance of $p<0.05$.

RESULTS AND DISCUSSION

In this work, the effect of inoculation time and spermidine in transformation efficiency was determined by measuring TGE in cell aggregates and the number of embryo colonies after the selection procedure. For the first variable, no significant differences were observed among samples treated and non-treated with spermidine, while a significant increase in TGE was provoked by inoculation times longer than 6 h, without differences between the 12 h and 24 h treatments Fifure 2, Table 1. A similar effect on TGE for this cultivar was previously reported by Arinaitwe *et al*. (2004) when increasing inoculation times from 4 h to 14 h. Apparently, longer inoculation times may favour the initial steps of chemotaxis, bacteria attachment and activation of *vir* genes during *Agrobacterium*-mediated transformation and increase TGE.

At the end of the two-month selection period, small embryo colonies were visible in the surface of the cell aggregates, while no embryo formation was detectable in untransformed controls placed on selective medium.

The spermidine-treated samples showed higher stable transformation frequencies when inoculation times were increased up to 12 and 24 h (Table 1), being the highest number of embryo colonies obtained with the 24 h+Spd treatment. The former treatment also showed no significant differences with untransformed controls. Furthermore, no differences among each other were found on the treatments without spermidine, while samples with and without the polyamine differed only when the inoculation times were increased. This suggests an additive effect of both, inoculation time and spermidine.

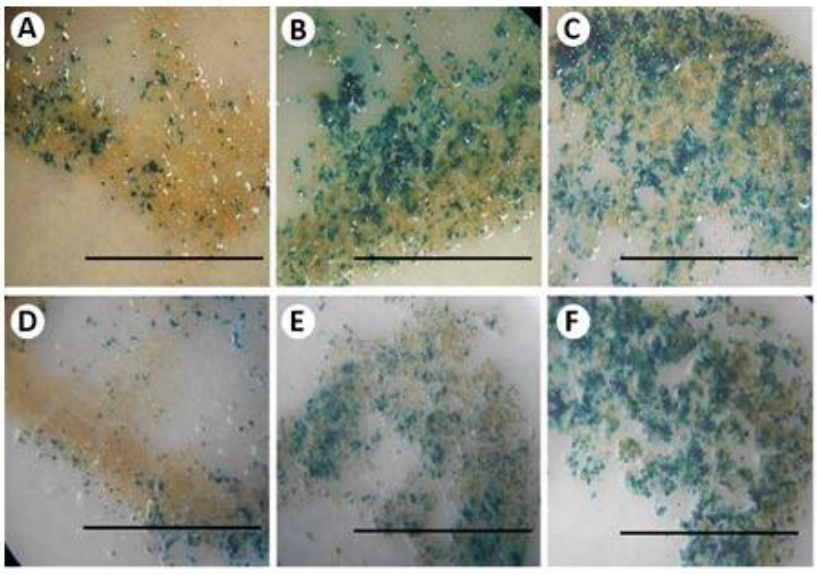

Figure 2. Effect of inoculation time and spermidine (Spd, 1 mM) on β-glucuronidase transient expression after the co-culture period in embryogenic cell aggregates of *Musa* cv. 'Grande naine' (AAA) previously inoculated with *Agrobacterium tumefaciens* for A, 6 h; B, 12 h; C, 24 h; D, 6 h+Spd; E, 12 h+Spd and F, 24 h+Spd (bar = 0.5 cm).

Table 1. Effect of spermidine (Spd, 1 mM) and time during inoculation of ECSs of *Musa* cv. 'Grande naine' (AAA) with *Agrobacterium tumefaciens* on β-glucuronidase transient expression (TGE) in cell aggregates after the co-culture period and the number of embryogenic colonies obtained after selection.

Treatment	TGE	Mean Rank	Number of embryo colonies	Middle rank
6 h	1540.73	149.4 b	12.63	44.62 d
6 h+Spd	1331.53	140.2 b	21.86	59.28 cd
12 h	2610.65	214.26 a	9.08	35.00 d
12 h+Spd	2186.56	183.63 a	27.11	67.66 c
24 h	2539.36	190.53 a	22.50	58.25 cd
24 h+Spd	2832.73	200.13 a	45.25	120.00 a
Untransformed control	-	-	37.67	97.00 ab
Untransformed control+Spd	-	-	31.90	82.00 b

Data show means and mean ranks of five independent experiments with six replicates of 200 µl at 33% settle cell volume of ECSs~50 mg fresh weight per treatment for the TGE and a single experiment with 15 replicates per treatment for the Number of embryo colonies. Means on the same column with different letters differ significantly ($p<0.05$) according to H of Kruskal Wallis and U of Mann Whitney Test.

A similar performance was reported by Khanna *et al*. (2004) during a heat shock treatment of 45°C for 5 min to 'Grande naine' and 'Lady Finger' (*Musa* AAB) ECSs before inoculation with *Agrobacterium*. The treatment did not increase the transient GFP expression, while it doubled the formation of embryo colonies. Moreover, Chong-Pérez *et al*. (2012 a) reported that 1 mM spermidine increased the number of embryo colonies and regenerated plants of cv. 'Dwarf Cavendish' (*Musa* AAA), as well as the TGE in embryogenic cell aggregates with an inoculation time of 6 h. The latter indicates that plant genotype might influence this response. Seemingly, spermidine has a protective effect on banana cell aggregates during *Agrobacterium* infection, which became more patent when increasing the inoculation time.

Is known that as a plant pathogen, *A. tumefaciens* elicits defense responses in plant tissues. Veena *et al*. (2003) described the transcriptional elicitation of defense genes in tobacco BY-2 cell cultures after inoculation with several *A. tumefaciens* strains. Also, in *Arabidopsis thaliana*, defense responses induced by the pathogen-associated molecular pattern EF-Tu reduce transformation by *A. tumefaciens* (Zipfel *et al*., 2006). A similar induction of defense genes have been reported for *Arabidopsis* cell cultures (Ditt *et al*., 2006) and inflorescence stalks (Lee *et al*., 2009), and wheat embryogenic calluses (Zhou *et al*., 2013) inoculated with *A. tumefaciens*. Among these responses, the occurrence of programmed cell death (PCD) is a major factor that negatively affects efficiency during *Agrobacterium*-mediated transformation of several plant species (Hansen 2000; Zhang *et al*., 2013). PCD can be triggered as a result of the oxidative burst in plant cells following interaction with the bacteria, where H_2O_2 acts as a signal and regulator (Kaurilind *et al*., 2015). Thus, a recent study showed that *Agrobacterium*-mediated H_2O_2 accumulation led to cell death during genetic transformation of tomato (*Solanum lycopersicum* L.) cotyledons (Dan *et al*., 2016). Also, the antioxidant defense systems and anti-PCD signaling cascades are inhibited during H_2O_2-induced PCD in tobacco cell suspensions (Vannini *et al*., 2012). Banana ECSs undergo apoptotic-like PCD after inoculation with *Agrobacterium* (Khanna *et al*., 2007), although whether cell death is triggered by H_2O_2 accumulation is still unknown.

Polyamines are related with PCD regulation (Moschou and Roubelakis-Angelakis, 2014) and spermidine, putrescine and spermine have been reported to modulate stress-triggered ROS homeostasis and oxidative damage (Moschou and Roubelakis-Angelakis, 2014; Sudhakar *et al*., 2015). There is growing evidence that polyamine accumulation is associated with induction of antioxidant enzymes like superoxide dismutase, catalase and ascorbate peroxidase, consequently reducing free radicals levels and oxidative

damage (Liu *et al*., 2015; Sudhakar *et al*., 2015). For instance, the exogenous addition of spermidine in tobacco (*Nicotiana tabacum*) cell suspensions protected the cells from heat-shock-induced PCD and rebounded the ascorbate peroxidase suppression (Marsoni *et al*., 2010). Similar results were obtained by this group when H_2O_2-induced PCD in tobacco cells was delayed by exogenous addition of spermidine (Vannini *et al*., 2012). This might explain the protective effect of spermidine observed in this study. Nevertheless, further studies are needed to elucidate the role of this polyamine during *Agrobacterium*-mediated transformation.

Additionally, of the 24 leaf fragments analysed for stable expression of the uidA gene, a total of 16 lines (66.6%) were positive for the GUS staining while the rest showed no coloration. The absence of GUS activity in these lines might be caused by several reasons: (i) the plants are not transgenic, (ii) the T-DNA is truncated or (iii) the *uidA* gene is silenced. In order to verify the previous conjectures, the presence of the genes *nptII* and *uidA* was analysed through PCR (Figure 3 b).

Fifteen lines were positive for the *nptII* and *uidA* fragments amplification, as well as for the GUS expression in leaves. However, lines 2, 3 and 4 (12.5%) were PCR negative, being escapes of the selective process. In addition, lines 1 and 16 were PCR positive for both genes but showed no GUS activity. This is probably due to the insertion of the T-DNA in a transcriptionally inactive region of the chromatin, in which case it should be considered as an escape. Also, the insertion of multiple copies of the transgenes might have negatively influenced the GUS expression (Rajeevkumar *et al*., 2015). Similarly, a deletion near the right border that affects expression but not the amplification might be a possible

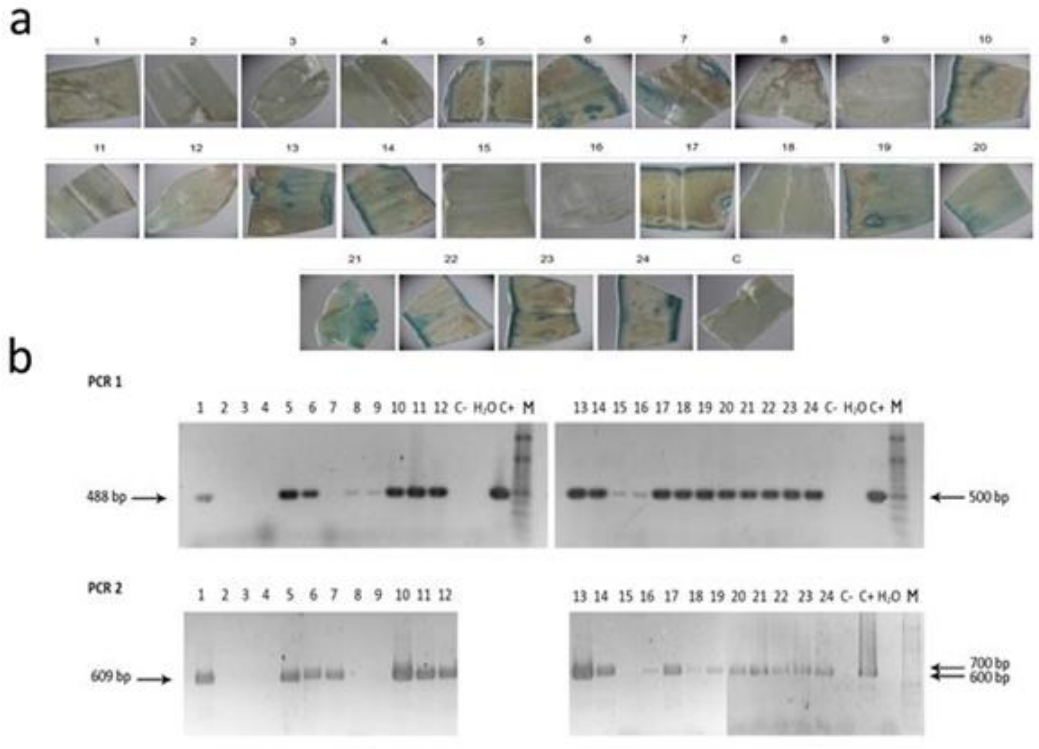

Figure 3. Analysis of 24 putatively transformed lines (1-4:6 h, 5-8:6 h+Spd, 9-12:12 h, 13-16:12 h+Spd, 17-20:24 h, 21-24:24 h+Spd) of the cv. 'Grande naine' (*Musa* AAA): a, histochemical detection of the GUS activity in leaf fragments, C, untransformed plants and b, ethidium bromide stained 1.5% agarose gel electrophoresis of the PCR products from DNA using specific primers for a 488 bp fragment of the *nptII* gene (PCR 1) and a 609 bp fragment of the *uidA* gene (PCR 2). C-, untransformed plants; C+, pFAJ3000 plasmid; H_2O instead of DNA; M, molecular weight marker O´Range RulerTM 100 bp DNA Ladder Plus (Fermentas, Germany).

explanation for this phenomenon. Furthermore, line 7 was positive for *uidA* amplification and GUS activity but resulted negative for *nptII*. On the contrary, lines 8 and 15 were *nptII* positive and *uidA* and GUS negative, probably as a result of the loss of the gene during the T-DNA transfer and integration processes (Ziemienowicz *et al*., 2008). Few years ago there was a consensus that T-DNA was preferentially inserted in transcriptionally active region of the plant genome (Chen *et al*., 2003), though this statement was based on the analysis of transgenic plants subjected to a selective process. Nevertheless, Kim *et al*. (2007) proved that T-DNA integration in the genome of *Arabidopsis* plants that have not been submitted to selection was a random process that included centromeric and telomeric regions. In the current study, three lines with putative deletions on the right border and one in the left border of the T-DNA were identified. This results were independent of the inoculation treatment used (data not shown) and suggest the occurrence of modifications on both borders of the insert in *Musa* sp., which is consistent with what was previously reported for this species by Pérez-Hernández *et al*. (2006 b).

CONCLUSIONS

This work shows that longer inoculation times in combination with spermidine can be used to enhance *Agrobacterium*-mediated transformation of the relevant banana cultivar 'Grande naine'. Also, it could have value for performing functional genomics analysis in *Musa* species. Finally, spermidine seems to protect the banana cells during infection with the bacteria.

ACKNOWLEDGEMENTS

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