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Genetic Transformation of the Banana Hybrid Cultivar 'FHIA-18' (*Musa* sp.) via Particle Bombardment using Argon Gas

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ABSTRACT

Bananas and plantains (Musa spp.) are extremely essential and important for about 400 million people in many developing countries across the world. In the Caribbean, both bananas and plantains are grown, but the banana cultivars are the most exported. Agriculture plays an important role in Belize's economy and remains a major driver for the economy's growth. The banana industry is the third export crop of Belize behind sugar cane and citrus and provides a significant base for employment and income generation in Belize. This work was carried out with the objective of studying parameters that influence the transient expression of the enzyme β-glucuronidase in cell suspensions of the banana hybrid cultivar 'FHIA-18' (Musa sp. AAAB). Studies were done with three distances and four pressures using a low pressure gene gun with argon gas. Results of this experiment revealed the importance of the distance and pressure of bombardment, with the most effective, being 9 cm and 140 psi respectively with significant difference to the other treatments. Different plasmid constructs were also studied and those under the control of the maize polyubiquitin promoter showed higher efficiencies than the CaMV 35S promoter. Biological factors are also of great importance and the physiological age of the cell suspensions confirmed its fundamental role in transformation efficiencies with the best results obtained when cell suspensions collected between 5 and 10 days after the last subculture were used. PCR and Southern blot analyses confirmed the presence of the transgene into the plant genome.

Key words: *Banana, Genetic transformation, Particle bombardment, β-glucuronidase, Argon gas.*

1. INTRODUCTION

In a world where the population growth is exceeding food production, agriculture and especially plant biotechnology, needs to be implemented rapidly in diverse aspects of life. Banana (*Musa* spp.) is among the most important crops in tropical and subtropical countries. World production between 2000 and 2015 of bananas grew at a compound annual rate of 3.7 percent, reaching a record of 117.9 million tons in 2015, up from around 68.2 million tons in 2000 (FAO, 2017). However, it is threatened by many pests and diseases, which create the need for the search of resistant cultivars.

Bananas are predominantly produced in Asia, Latin America and Africa. The biggest producers are India, which produced 29 million tons per year on average between 2010 and 2015, and China at 11 million tons (FAO, 2017). The production of the hybrid cultivar 'FHIA-18' (*Musa* sp. AAAB) has extended in Latin America and its popularity and acceptance has grown in most countries in this region.

Conventional breeding programs in banana are difficult because of its sterility and polyploidy. Genetic transformation is one potential strategy in developing new varieties in a relatively short period of time. However, the success of this method depends on an efficient plant regeneration system. For genetic transformation, the ideal model is using isolated embryogenic cells cultivated in suspension and its subsequent regeneration via somatic embryogenesis, minimizing the formation of chimeras (Jimenez, 1998).

The objectives of this research is to study important parameters like the pressure and distance to carry out the particle bombardment, as well as the plasmid construct. Other important biological parameter like the age of the cell suspension is also studied in this work.

2. MATERIALS AND METHODS

2.1 Study of pressure and distance of bombardment.

Embryogenic cell suspensions established from young male flowers of the hybrid cultivar 'FHIA-18' (*Musa* sp. AAAB) (Gomez *et al.*, 2000) were bombarded with a low pressure gene gun (CINVESTAV, 2000) using argon gas. The purification of all plasmids was done according to Sambrook *et al*. (1989). Tungsten particles M-10 (Bio-Rad) with an approximate diameter of 0.7 μm were used in all the experiments. Disinfection and coating of the microparticles with the DNA was carried out according to Sanford *et al.* (1993). The different gas pressures studied were 120, 130, 140 and 150 psi and the distances were 6, 9, 12 and 15 cm from the biological target. After bombardments, the cells were then cultured in 9-cm diameter Petri dishes, which contained semisolid culture medium composed of MS salts and vitamins, 0.5 $mg.L^{-1}$ biotin, 100 $mg.L^{-1}$ L-glutamine, 100 $mg.L^{-1}$ malt extract, 13.5 μM 2,4-D and 45 $g.L^{-1}$ sucrose. These Petri dishes were then placed in total darkness at a temperature of 27 ± 2.0°C. The culture medium was solidified with 2.3 $g.L^{-1}$ of Phytagel and its pH was adjusted to 5.3 prior to sterilization. Three days after bombardment, histochemical assays with X-Gluc (Jefferson, 1987) were done to evaluate transient β-glucuronidase (GUS) expression.

2.2 Different plasmid constructs.

Five different plasmid constructs were studied to evaluate the effects of the promoter of the constructs. The plasmids used in this study were pAHC25 (9.7 kb), pBPF-A5-GUS (5.637 kb), pUGCI (6.86 kb), pCAMBIA3301 (5.077 kb) and pBPF-U5 (6.37 kb). The best result with respect to the pressure and bombardment distance from the previous experiment was used in this study. Twelve replicas were used per treatment and following bombardment, the cells were placed in Petri dish of 9 cm diameter, which contained the semisolid culture medium previously described in this work. Transient GUS expression was evaluated after staining with X-Gluc 3 days after bombardment.

2.3 Study on the amount of microparticle and number of bombardment per sample

After the previous parameters have been studied and optimized, this experiment was done to evaluate the effects of the amount of microparticle per bombardment, as well as the number of bombardments per sample. Aliquots of 7.0, 9.0 and 11.0 μL of the microparticle suspension coated with the DNA were studied, as well as one and two bombardments per sample. After bombardments, cells were cultured as previously described and 3 days thereafter transient GUS expression was evaluated.

2.4 Effects of the age of the cell suspensions

Biological factors play a very important role in the success of genetic transformation. Cell aggregates collected from the fifth, tenth and fifteenth day after the last subculture of the suspensions were used for this experiment. All the parameters previously studied and established were used to carry out this experiment. After bombardments, cells were cultured as previously described and 3 days thereafter, transient GUS expression was evaluated.

2.5 Selection and regeneration of plants

After optimizing the parameters previously studied, cell aggregates from the banana hybrid cultivar 'FHIA-18' were transformed with the plasmid pAHC25. After carrying out 50 separate bombardments, cells were cultured as described earlier for 3 days in order to recover from the impact of the microparticles. Thereafter they were passed to another culture medium with similar components with the exception of L-glutamine and the inclusion of the selective agent, which was 6.0 $mg.L^{-1}$ of the herbicide BASTA. These cells were cultured under these conditions for eight weeks with subculture and a change of culture medium every two weeks.

After this period, the surviving cells were passed to a culture medium proposed by Bieberach (1995) for the formation of somatic embryos. This medium was composed of SH salts, MS vitamins, 0.5 $mg.L^{-1}$ biotin, 100 $mg.L^{-1}$ malt extract, 100 $mg.L^{-1}$ L-glutamine, 230 $mg.L^{-1}$ L-proline, 1.1 μ*M* naphthaleneacetic acid (NAA), 0.98 μ*M* 2-isopentenyladenine (2ip), 0.23 μ*M* kinetin, 10 $g.L^{-1}$ lactose, 0.23 μ*M* zeatin, and 45 $g.L^{-1}$ sucrose. Somatic embryos in the globular stage formed in this phase were matured in the following culture medium: MS salts and vitamins, 2.22 μ*M* N6-benzylaminopurine (BA), 2.85 μ*M* indole-3-acetic acid (IAA), 100 $mg.L^{-1}$ myoinositol, and 30 $g.L^{-1}$ sucrose (Gómez *et al.*, 2000). Somatic embryos were germinated in the following culture medium: MS salts and vitamins, 2.22 μ*M* BA, 11.4 μ*M* IAA, 100 $mg.L^{-1}$ myoinositol, 0.01 $mg.L^{-1}$ Biobras-6 (a plant growth promoter developed by *Centro de Productos Naturales* of the University of Havana), and 30 $g.L^{-1}$ sucrose (Gómez *et al.*, 2000). For germination, glass vessels of 250 mL capacity were used, which contained 30 mL of culture medium. Ten somatic embryos were placed in direct contact with the medium in

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each vessel. These cultures were placed in a special growth chamber which allows the penetration of indirect solar light at an illumination intensity of 50-62.5 $\mu mol.m^{-2}.s^{-1}$, a 12-h photoperiod and a temperature of 27 ± 2°C. Each plant regenerated was considered an individual line.

2.6 PCR and Southern blot analysis

Seventy independent putative transgenic lines were regenerated. PCR was carried out on these lines to confirm the integration of the transgene into the plant's genome. Genomic DNA was extracted from the plants using the following procedure: 100 mg of tissues from young leaves of *in vitro* plants were ground in liquid nitrogen until a fine powder was obtained; 500 µL of extraction buffer [500 mM NaCl, 100 mM Tris/HCl (pH 8.0), 50 mM EDTA, 2% (w/v) polyvinyl pyrrolidone (PVP) and 10 mM β-mercaptoethanol] were added; 33 µL of sodium dodecyl sulphate at 20% was added and samples were vortex and incubated at 55°C for 10 min; after this period 160 µL of potassium acetate (5 M final concentration) was added, vortex and centrifuged at 13 600 x g for 10 min to remove cell debris; 700 µL of cold isopropanol were added to precipitate the nucleic acids at -20°C during 5 min; centrifuge at 13 600 x g for 10 min; 200 µL of ethanol at 70% was added to the precipitate; this was centrifuged again under the same conditions previously mentioned, dried and re-suspended in 40 µL of water; RNAse was added and left for 30 min. at 37°C then stored at 4°C.

Polymerase chain reactions were performed in 200-µL PCR tubes using a Mastercycler Gradient PCR machine. Reaction mixtures for DNA amplification included 200 µM of dNTPs, 1.0 U of *Taq* DNA polymerase (Qiagen) in 1 X PCR buffer (Qiagen), 1.5 µL of $MgCl_2$ at final reaction volumes of 25 µL. The primers used were: Oligonucleotide bar 5' 5' CGA GAC AAG CAC GGT CAA CTT C 3' Oligonucleotide bar 3' 5' GAA ACC CAC GTC ATG CCA GTT C 3'.

PCR conditions included an initial denaturalization step of 96°C during 2 min. followed by a temperature of 94°C for 1 min. Annealing took place at 62°C for 1 min. and extension was at 72°C for 1.5 min. Thirty-five cycles were carried out with a final elongation step at 72°C for 7 min. then cooled to 4°C.

Amplified fragments were separated by electrophoresis for 20 min. at 120 V on 1.2% (w/v) agarose gels in 1 X TBE buffer (40 mM Tris-acetate, 1 mM EDTA). To load samples on gels, 5 µL volumes of PCR reactions were mixed with 1 µL loading buffer [0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 40% (w/v) sucrose].

For the Southern blot analyses, genomic DNA was isolated and digested with the restriction enzyme EcoRI. The digested DNA was separated on a 0.8% agarose gel and subsequently transferred to a nylon membrane (Boehringer Mannheim) by the method of Southern (1975). Hybridization was done with a [^{32}P]-labeled probe. Membranes were washed as recommended by the membrane supplier.

Data collected from the experiments were processed with the statistical program, SPSS/PC for Windows, as well as with STATGRAPHICS plus for Windows. Comparison means tests used to determine significantly different groups were Duncan and Dunnetts'C.

3. RESULTS AND DISCUSSIONS

3.1 Study of pressure and distance of bombardment.

The results of this experiment show that the pressure and distance of bombardment play an important role in the expression of the reporter *gus*A gene that encodes the enzyme β-glucuronidase and it is indispensable to optimize these factors before establishing a genetic transformation protocol for banana. The best pressure and distance combination was 140 psi and 9 cm respectively. This treatment produced a total of 56.8 blue foci per sample with significant difference to all the other treatments (Table 1).

Table 1. Number of blue foci per shot at different distances and pressures in the banana hybrid cultivar 'FHIA-18' (*Musa* sp. AAAB).

Bombardment distance (cm)	Pressure (psi)			
	120	130	140	150
6	23.4 fg	27.9 ef	36.7 cd	10.8 h
9	24.4 fg	39.8 c	56.8 a	13.0 h
12	23.6 fg	31.8 de	46.1 b	28.0 ef
15	13.2 h	23.5 fg	36.8 cd	39.7 c

Values marked with distinct letters differ statistically for p<0.05 according to Duncan.

A pressure of 120 psi of argon gas resulted insufficient for the penetration of the microparticles into the cells and hence caused a low expression of β-glucuronidase. However, 150 psi was a pressure too high, which caused significant cell damage due to the impact of the microparticles.

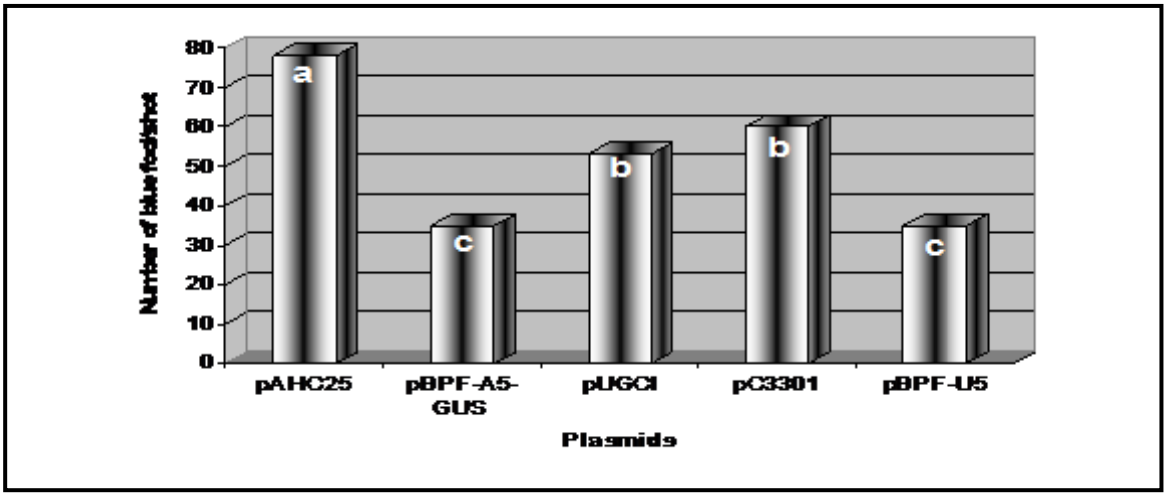
Daniels *et al*. (2002) conducted similar studies of these parameters, however, on the plantain hybrid cultivar FHIA-21. They reported that the best pressure was 140 psi; however, at a longer distance of 12 cm. Ságі *et al.* (1995a) used pressures from 43 to 87 psi and obtained transient gene expression in the cultivar 'Bluggoe' (*Musa* sp. ABB). In these experiments, these authors used a gene gun built based on the description of Takeuchi *et al.* (1992) using helium as the propulsive gas.

Transformation efficiencies vary with different plant species and the design of the gene gun (Rey *et al.*, 1996). Becker *et al.* (2000) selected a distance of 7.5 cm and a pressure of 80 psi for the bombardment of cell suspensions in the cultivar 'Grand Naine' (*Musa* sp. AAA), while Grapin (1995) obtained best results at a distance of 10-13 cm in different cultivars of *Musa*. All these authors used helium gas in all the cases previously mentioned.

3.2 Different plasmid constructs.

For all plant species and each type of explant, it is necessary to identify highly expressive promoters, which have specific tasks in the introduction and integration of the transgene in the genome of the plant. In this experiment, the plasmids that produced the greatest number of blue foci per sample was pAHC25, which was under the control of the maize polyubiquitin promoter. This result was significantly superior to the other plasmids studied (Figure 1).

Figure 1. Number of blue foci per shot with five different plasmids in the banana hybrid cultivar 'FHIA-18' (*Musa* sp. AAAB)



Values marked with distinct letters differ statistically for p<0.05 according to Duncan.

Elayabalan & Kalaimughilan (2013) transformed cells from *Musa* spp. using particle bombardment with three different plasmids containing the uid-A gene. This resulted in a strong GUS expression five days after bombardment; however, plant regeneration from bombarded cells was much lower than non-bombarded ones.

According to Ságі *et al.* (1995b) the maize polyubiquitin promoter is one of the strongest for *Musa* spp. These authors obtained the best results in *Musa* spp. with the plasmid pAHC27 under the control of the same promoter. Grapin (1995) studied different plasmids in cultivars like 'French Sombre' (AAB), 'Grand Naine' (AAA) and *M. malaccencis* (AA) and found that in all cases, the best result was obtained with the plasmid pUGCI. However, this study showed that for FHIA-18 the best plasmid proved to be pAHC25 with significant differences when compared to the other plasmids studied.

Liu *et al*. (2016) who worked with five different *Musa* varieties, reported that in the Gongjiao variety, GUS expression was detected in almost 10 out of every 100 explants (10%). Because the pCAS04 construct used in transformation experiments contained a promoterless GUS, GUS expression in the putative transformants indicated genomic insertion of the construct downstream from a functional promoter. Therefore, GUS staining could be used to identify transgenic plants

3.3 Study on the amount of microparticle and number of bombardment per sample

The results obtained in this experiment show that the amount of microparticles used to carry out each bombardment, doesn't affect the transformation efficiency. Carrying out one bombardment on the cell aggregates resulted in values of blue foci significantly superior than to the treatments with two bombardments on the same sample (Table 2). Two bombardments had a negative effect, which could be imputed to the fact that the cells suffered great damage on being bombarded twice since tungsten microparticles have an irregular shape. Also Sanford *et al.* (1993) suggested that tungsten microparticles were potentially toxic to certain types of cells, which could cause direct damage or by means of acidification of the culture medium.

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Table 2. Effect of the amount of microparticles per shot and the number of shots per sample on cell aggregates of the banana hybrid cultivar 'FHIA-18'.

Treatment	Amount of microparticles (µL)	Number of shots	Number of blue foci
1	7	1	67.50 ± 5.06 a
2	9	1	70.30 ± 4.14 a
3	11	1	73.80 ± 2.92 a
4	7	2	36.60 ± 3.64 b
5	9	2	32.20 ± 2.82 b
6	11	2	18.40 ± 1.21 c
	M ± SE		49.80 ± 3.12

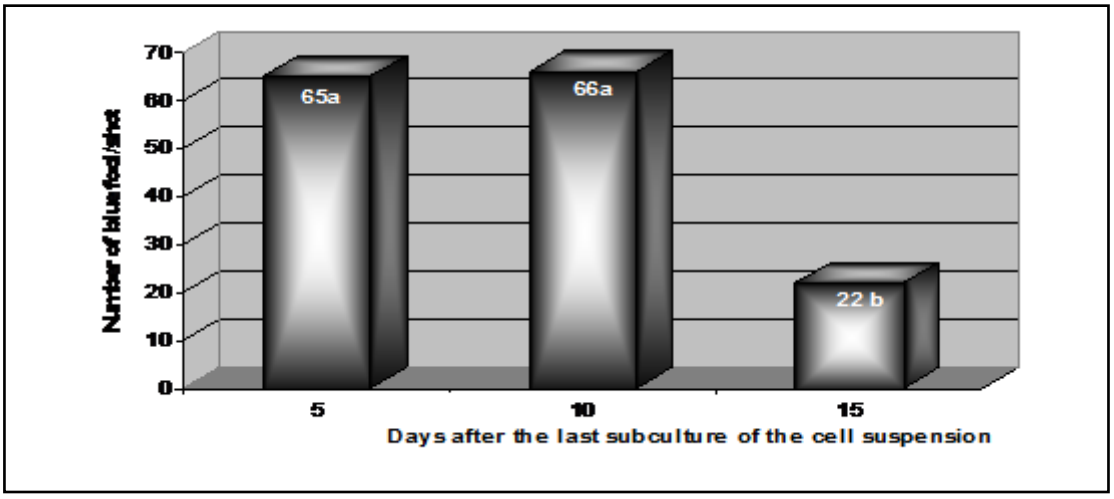
Values marked with distinct letters differ statistically for p<0.05 according to Dunnett'C.

Klein *et al.* (1988) observed that GUS expression reached its highest point when small quantities of microparticles were used to transform maize, while increasing the amount to 5.0 µL resulted in a drastic decrease in the expression units observed. On the contrary, Becker *et al.* (2000) used aliquots of 5.0 µL for each bombardment on cell suspensions of the cultivar 'Grand Naine' (*Musa* sp. AAA). In this same cultivar, Más *et al.* (2000) obtained the greatest efficiencies using 12 µL of tungsten microparticles on somatic embryos in the globular stage. This factor depends on the plant species and the type of explant used.

3.4 Effects of the age of the cell suspensions

The age of the cell aggregates to be bombarded is very important and based on the findings of this study plays a vital role in the success of the transformation. Using cell aggregates collected at five and ten days after the last subculture, values of 65 and 66 blue foci were observed respectively with significant differences to that observed using cell aggregates 15 days after the last subculture (Figure 2). This result could be attributed to the fact that 15 days after the last subculture, the cells are no longer in the logarithmic growth phase and hence are not in active cell division.

Figure 2. Effect of the age of the cell suspension after the last subculture on transformation efficiency in the banana hybrid cultivar 'FHIA-18' (*Musa* sp. AAAB).



Values marked with distinct letters differ statistically for p<0.05 according to Dunnett'C.

Ságі *et al.* (1995a) pointed out that transient gene expression reached its highest point when cell suspensions of 5 and 6 days old in the cultivar 'Bluggoe' were used. While Becker *et al.* (2000) used cells collected four days after the last subculture in the cultivar 'Grand Naine'.

3.5 Selection and regeneration of plants

Cells cultured in the selection culture medium started showing necrosis after one week of culture. This necrosis further resulted in cell death, which was due to the effects of the selective agent, BASTA. After eight weeks under these conditions, only a few cells showed signs of survival and these were regenerated into plants. A total of 70 individual plants were regenerated, which represented an average of 1.4 putative transgenic plants per sample. In *Musa*, Becker *et al.* (2000) regenerated 0.1 individual plants per sample, while Ságі *et al.* (1995b) obtained 2.7 putative transgenic plants per sample.

3.6 PCR and Southern blot analysis

Results from the PCR showed the bands from the putative transgenic lines analyzed which coincide with the positive control (pAHC25). Also no band appeared for the negative control in lane eight which was an untransformed plant nor in lane 9 (Figure 3), which proves that there was no contamination during the reaction. Southern blot hybridization of five plants transformed with pAHC25 confirmed the stable integration of the *bar* gene into the genome. The hybridization signals in lanes containing genomic DNA of transgenic plants were of different molecular weights to the positive control plasmid, whereas no signal was detected in the non-transformed control lane. The copy number ranged from 1 to 4 based on the number of bands present in each lane (Figure 4). The presence of multiple copies of the introduced gene is common among transgenic plants obtained via microparticle bombardment (Altpeter *et al*., 1999).

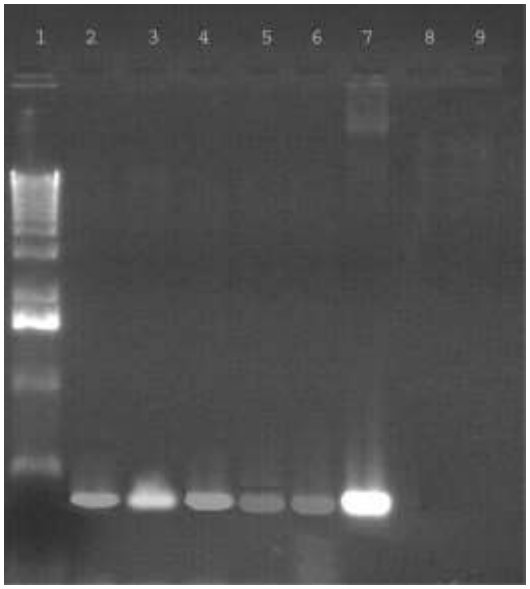


Figure 3. Result from the PCR. Lane 1. molecular weight marker. Lanes 2-6. Transgenic plants. Lane 7. Positive control plasmid (pAHC25). Lane 8. Negative control, which was a non-transformed plant. Lane 9. Negative control, which was without genomic DNA.

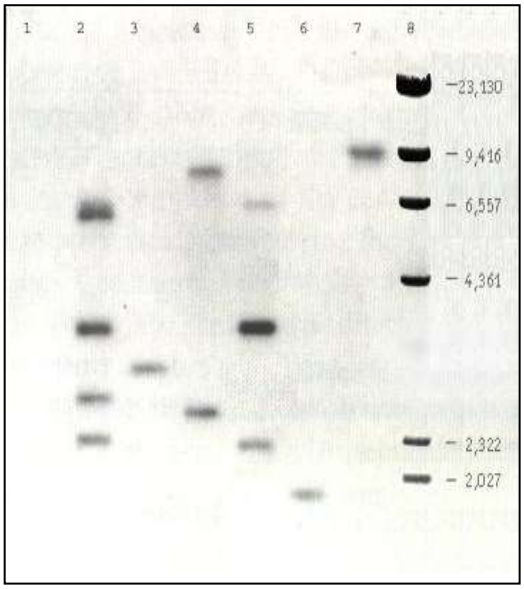


Figure 4. Southern blot analysis of five putative transformed plants. Lane 1. Negative control, which was a non-transformed plant. Lanes 2-6. Five putative transformed plants. Lane 7. Positive control plasmid (pAHC25). Lane 8. Molecular weight marker.

4. CONCLUSIONS

The best bombardment efficiencies were obtained with a distance of 9 cm and a pressure of 140 psi, which was significantly superior to the other treatments tested. The plasmid pAHC25, under the control of the maize polyubiquitin promoter had the highest transient GUS expression. The amount of microparticle studied didn't influence the results of the GUS expression; however, two bombardments on the same sample had a negative effect on the results obtained. Cell aggregates collected 5 to 10 days after the last subculture resulted ideal for its use in transformation of this hybrid cultivar. PCR and Southern blot analyses proved the stable integration of the *bar* gene into the genome of the plants.

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