African Journal of Biotechnology Vol.11(24), pp. 6612-6618, 22 March, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.087
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Callus induction, regeneration and transformation of sugarcane (*Saccharum officinarum* L.) with chitinase gene using particle bombardment

Rattana Khamrit[1], Prasit Jaisil[2] and Sumontip Bunnag[1]*

[1]The Applied Taxonomic Research Center (ATRC), Department of Biology, Faculty of Science, Khon Kaen University, Khonkaen 40002, Thailand.
[2]Department of Agronomy, Faculty of Agriculture, Khon Kaen University, Khonkaen 40002, Thailand.

Accepted 2 March, 2012

This study was carried out to optimize the conditions for introducing a chitinase gene into the sugarcane cv. Phil 66-07 calli by particle bombardment. Young leaves were cultured on the modified Murashige and Skoog (MS) medium supplemented with varied concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA), yeast extract and coconut water (CW). The maximum percentage of callus induction was obtained from the MS medium supplemented with 3 mg/l 2,4-D and 15% (v/v) coconut water. Multiple shoots were achieved by transferring sugarcane calli to the MS medium amended with 1 mg/l benzyl aminopurine (BA) and 0.5 mg/l indole-3-butyric acid (IBA). Additional experiments were also performed to determine the effect of antibiotics on regeneration of sugarcane. It was found that growth of sugarcane calli and plantlets were completely inhibited by hygromycin concentrations of 25 and 50 mg/l, respectively. The genetic transformation was achieved via particle bombardment with an optimal helium pressure of 900 psi and the stopping screen set at 9 cm. Sugarcane was transformed with either GUS or a chitinase gene and a gene for hygromycin selection. GUS transformed calli were produced to optimize the particle bombardment protocol. Using the optimized protocol, the chitinase gene was transformed into sugarcane and polymerase chain reaction (PCR) was used to verify the integration of a chitinase gene, 35S promoter and nitric oxide synthase (NOS) terminator in transgenic sugarcane.

Key words: Sugarcane, genetic transformation, particle bombardment, chitinase gene.

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is one of the most important cash and industrial crop and is widely cultivated in tropical and subtropical countries of the world for sugar and bioethanol production in a total of 19.4 million hectors as a single crop (Raza et al., 2010; Imtiaj et al., 2007). It accounts for approximately 80% of the world's sugar production (Raza et al., 2010). In Thailand, sugarcane production is much lower than most of the sugarcane growing countries of the world. The low cane and sugar yields are attributed to several factors where salinity, drought, pests and diseases constitute major constraints (Nasir et al., 2000; Khaliq et al., 2005). The major pests and diseases that cause losses in sugarcane production include canegrubs, feral pigs, ratoon stunting diseases (RSD), sugarcane rusts, chlorotic streak and soil-borne diseases (McLeod et al., 1999).

Approximately 100 diseases of sugarcane have been reported from different parts of the world which hinder sugarcane growth (Khurana and Singh, 1975), thus lowering sugar production. Fungal diseases in sugarcane are the most predominant diseases appearing as spots

*Corresponding author. E-mail: sumbun@kku.ac.th. Tel: 66 81 8074700. Fax: 66 43 364169.

Abbreviations: MS, Murashige and Skoog; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **NAA,** naphthalene acetic acid; **CW,** coconut water; **BA,** benzyl aminopurine; **IBA,** indole-3-butyric acid; **NOS,** nitric oxide synthase.

Table 1. Varied concentrations of 2,4-dichlorophenoxy acetic acid (2, 4-D), naphthalene acetic acid (NAA), coconut water and yeast extract contained in the Murashige and Skoog (MS) medium used for callus induction.

MS	Medium					
	C1	C2	C3	C4	C5	C6
2,4-D (mg/l)	3	3	3	0	0	0
NAA (mg/l)	0	0	0	3	3	3
Coconut water (%)	0	15	0	0	15	0
Yeast extract (%)	0	0	20	0	0	20

Table 2. Varied concentrations of hormones used for plant regeneration.

Hormone (mg/l)	Medium					
	S1	S2	S3	S4	S5	S6
BA	0	0.5	1	2	3	4
IBA	0	0.5	0.5	0.5	0.5	0.5

on the leaves. These spots prevent the vital process of photosynthesis to take place, thus affecting growth and consequently the yield (Patil and Bodhe, 2011). The applications of smokes and fungicide are among the most common methods to control fungal diseases in Thailand; however, these methods are laborious and the chemicals used may endanger the environment for many years. With the advent of biotechnology and genetic engineering, efforts to create plants with favorable traits have been made worldwide. Chitinase is a glycosyl hydrolase that catalyzes the hydrolysis of β-1,4-glycosiidic bonds in chitin, which is a major component of the fungal cell wall. The chitinase could be detrimental to fungi. The continual expression of chitinase in plants could prevent insect and fungi damage (Kramer and Muthukrishnan, 1997). Creating sugarcane varieties resistant to fungal diseases by genetic transformation with a chitinase gene is an alternative means to prevent fungal damage.

This work reports on the improvement of sugarcane cv. Phil 66-07, which is the commercially important crop of Thailand with high sensitivity to fungi, by transforming it with a chitinase gene via particle bombardment.

MATERIALS AND METHODS

Plant materials

Apical regions of sugarcane (*S. officinarum* L.) cv. Phil 66-07 stems aged 5 to 6 months were used as explants. Outer leaves were removed to obtain the six inner leaves. A 10 cm long leaf roll possessing apical meristems was sterilized in 70% alcohol for 3 min, soaked in 10% sodium hypochlorite solution for 20 min, and then washed five times in sterile distilled water. The remaining leaves were aseptically removed until the three or four leaves were completely obtained. At this point, small transverse sections (2-3 mm) of the leaf roll were excised 10 cm or less, above the apical meristems and used as explants.

Callus induction

Explants were cultured on the MS (Murashige and Skoog, 1962) basal medium containing different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA), yeast extract and coconut water (Table 1), 30 g/l sucrose, 8 g/l agar, pH of 5.7 before autoclaving. The cultures were kept in the dark at 25±2°C for two weeks. After this period, calli were cultured in the light at 25±2°C for two weeks. The cultures were evaluated for number of explants producing calli and weight of calli to determine the optimal concentrations of 2,4-D, NAA, yeast extract and coconut water for callus induction. Each experiment was arranged in a randomized design with five replicates.

Plant regeneration

Calli were transferred to the MS medium supplemented with varied concentrations of 6-benzylaminopurine (BA) and indole-3-butyric acid (IBA) (Table 2), 30 g/l sucrose, 8 g/l agar and 500 mg/l casein hydrolysate, pH of 5.7. The cultures were incubated at 25±2°C with 16/8 h light/dark photoperiod. The cultures were then evaluated for number of callus formation, shoots and number of shoots per callus. All experiments were arranged as completely randomized design with ten replicates.

Effect of antibiotic

Optimal concentration of hygromycin required to inhibit growth of calli and plantlets was determined. Calli, 3 mm diameter were plated into callus induction medium containing varied concentrations of hygromycin (0, 10, 20, 30, 40, 50, 60 and 70 mg/l), while plantlets, 3-5 cm tall, were plated into regeneration medium containing varied concentrations of hygromycin (0, 25, 50, 75 and 100 mg/l).

The cultures were then kept at 25±2°C with 16/8 h light/dark photoperiod for four weeks. The optimal concentrations of

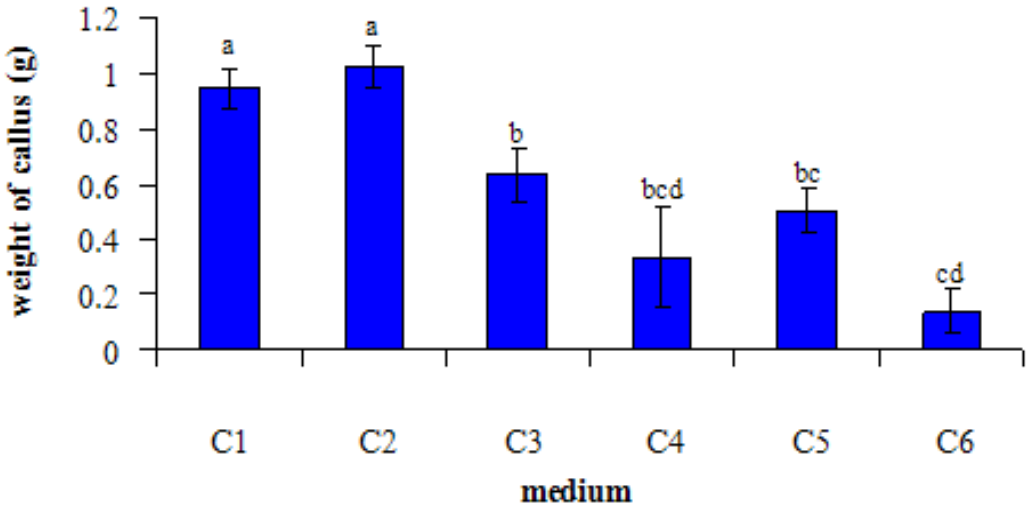

Figure 1. Average weights of calli induced from young leaves of sugarcane cv. Phil 66-07 on the MS medium containing different concentrations of 2,4-D, NAA, yeast extract and coconut water for 4 weeks.

antibiotics required to select transformed-calli or plantlets were determined.

Particle bombardment

Thirty milligram (30 mg) of 1.1 µm tungsten particles were transferred into a microcentrifuge tube and mixed with a Vortex mixer for 5 min in 0.5 ml of 70% (v/v) ethanol. Then, the suspension was held at ambient temperature for 15 min, centrifuged at 10,000 g for 5 s, decanted and washed tree times with 0.5 ml of sterile distilled water before suspending in 50 µl of sterile 50% (v/v) glycerol. The suspended particles were then coated with plasmid DNA by adding 5 µg DNA, 50 µl of 2.5 M $CaCl_2$ and 20 µl of 0.1 M spermidine (base-free form). The mixture was then thoroughly mixed with a Vortex mixer for 3 min, held at ambient temperature for 5 min and washed sequentially with 70% (v/v) ethanol and absolute ethanol before being resuspended in 48 µl of absolute ethanol. The DNA-coated particles were then mixed for 3 s before placing 8 µl of aliquots on macrocarriers for bombardment (Sanford et al., 1993). The calli were placed in the center of petridishes containing callus induction medium.

These calli were then bombarded with the DNA-coated tungsten particles. The distances from the stopping screen to targets were 6, 9 and 12 cm, and the rupture disc pressures were 900 and 1,100 pounds per square inch (psi).

Histochemical GUS assay

GUS expression assay were performed 15 days after bombardment. Calli were soaked in 2.0 mM 5-bromo-4-chloro-3-indole-β -D-glucuronide (x-gluc) and incubated for 24 h at 37°C. Thereafter, blue cells were counted as described by Jefferson (1987).

PCR analysis

Total DNA was extracted from transformed plantlets and non-transformed (control) plantlets using CTAB method (Doyle and Doyle, 1987). The primer sequences for PCR were as follows: 35S forward sequence (F) 5'-GCTCCTACAAATGCCATCA-3', reverse sequence (R) 5'-GAT AGTGGGATTGTGCGTCA-3'; NOS (F) 5'-GAATCCTGTTGCCGGTCTTG-3', (R) 5'-TTATCC TAGTTTGC-GCGCTA-3'; chi (F) 5'-CTCCATCATATCCCCCTC-3', (R) 5'-ATCCAGAAC CAGAACGCC-3'. Reactions were performed with the standard program which was comprised of one cycle of 3 min at 94°C, followed by 40 cycles of 40 s at 55°C for 35 s and NOS primers or 60°C for chi primer, 1 min at 72°C and ended with one cycle of 3 min at 72°C. PCR products were size-separated by electrophoresis on a 1.5% agarose gel with ethidium bromide at 50 V for 90 min.

RESULTS AND DISCUSSION

Callus induction

Callus initiation could be seen from the cut surface of young leaves in all treatments after one week of culture. Two types of calli (compact and friable) were recognized. Compact calli are bright yellow clumps, while friable calli, which are optimal for transformation are whitish clumps. These two types of calli have also been reported by Abdulla et al. (2002) and Honda et al. (1999). The highest callus induction percentage of 100 and the maximum weight of callus of 1.02±0.0758 g were obtained from which explants were cultured on the MS medium supplemented with 3 mg/l 2,4-D and 15% (v/v) coconut water (Figure 1). However, there was no significant difference between the addition and non-addition of coconut water into the medium. These findings are in agreement with Gandonou et al. (2005) and ElYacoubi et al. (2010) reporting that calli of sugarcane cultivars were formed when medium was supplemented with 3 mg/l 2,4-D. Furthermore, Begum et al. (1995) found that 3-5 mg/l 2,4-D produced high percentage of callus in five

Table 3. Percentage of regeneration and number of shoots per callus of sugarcane cv. Phil 66-07 cultured on the regeneration medium.

Medium	% Regeneration	Number of shoots/callus
S1	60	1.8
S2	100	16.5
S3	100	39.1
S4	100	24
S5	100	13.7
S6	80	4.8

sugarcane varieties, while Wagih and Adkias, (1999) reported that 2,4-D up to 6 mg/l reduced callus fresh mass.

Plant regeneration

Green cells within the callus were observed within one week of culture. Normal stems and leaves were produced from these cells. Calli exhibited shoot formation and multiplication the best when cultured on the medium supplemented with 1 mg/l BAP and 0.5 mg/l IBA. The highest percentage of shoot regeneration was 100, and the maximum number of shoots per callus was 39.1 (Table 3). However, Karim et al. (2002) found that the MS medium supplemented with 1 mg/l BAP and 0.5 mg/l IBA performed the best for shoot formation in sugarcane variety Isd-31. Furthermore, Khan et al. (2009) studied the effect of cytokinins on shoot multiplication in the sugarcane varieties HSF-240, CP-77-400 and CPF-237, and found that the optimum multiplication for variety HSF-240 was obtained at 1.5 mg/l BAP, 0.5 mg/l Kin with 16.5 cm shoot length, 11 numbers of tillers and 32 numbers of leaves per plant. Similarly optimum multiplication for variety CP-77-400 was obtained at 1.0 mg/l BAP and 0.5 mg/l Kin, with a maximum of 8.5 cm shoot length, 7 numbers of tillers and 24 numbers of leaves. Best multiplication rate for variety CPF-237 was observed at 1.0 mg/l BAP and 0.1 mg/l Kin with a maximum of 12 cm shoot length, 6 numbers of tillers and 18 leaves per plant. Tarique et al. (2010) determined the optimal concentrations of BAP with NAA or IBA for shoot initiation and multiplication of the sugarcane varieties Isd-16, Isd-36 and Isd-37, and found that 1.0 mg/l BAP + 0.5 mg/l NAA showed the best result for induction and multiplication of shoot. Mamun et al. (2004) studied the optimal concentrations of plant growth regulators on shoot proliferation of the sugarcane varieties Isd-28 and Isd-29, and found that 1.5 mg/l BA produced high percentage of shoot proliferation. Isd-28 and Isd-29 showed best shooting when 1.5 mg/l BA and 0.5 mg/l NAA were applied, respectively. Combinations of phytohormones often determine the course of morphogenesis such as shoot organogenesis and embryogenesis (Yataka et al., 1998).

Effect of antibiotic

Antibiotics were used in the present study in order to select transformed plantlets. Normally, non-transformed calli and plantlets are able to develop on medium in the absence of hygromycin. Based on the findings, the lowest concentrations of hygromycin that completely inhibited callus and plantlet growths were 25 and 50 mg/l, respectively (Figure 2). All calli and plantlets turned brown and eventually died within 3 weeks. Reynaerts et al. (1988) recommended the usage of hygromycin as a selectable marker as it is more toxic than kanamycin and kills sensitive cells more quickly. Angenon et al. (1994) reported that the selection agent should fully inhibit growth of untransformed plant cells; therefore, the lowest concentration of the selection agent that suppresses growth of untransformed cells is generally used. Hygromycin is widely used as a selectable marker in many plant species such as wheat (Lifang et al., 2001) and *Phalaenopsis* orchids (Sjahril et al., 2006).

Histochemical GUS assay

Histochemical GUS assay was carried out to detect expression and enzyme activity of GUS in transgenic calli. GUS gene is a reporter gene that encodes for β-glucuronidase enzyme and acts on the substrate X-gluc, producing blue color in transgenic calli. Non-transformed calli did not show blue color, whereas transgenic calli exhibited blue coloration. The results reveal that the optimal helium pressure for sugarcane was 900 psi (Figure 3), while the optimal distance from stopping screen to sugarcane calli was 9 cm (Figure 4). Maneewan et al. (2005) found that rice transformation via particle bombardment at the distance of 9 cm from stopping screen to callus yielded the highest expression percentage of 100.

PCR analysis

DNA isolated from transformed and non-transformed plants, and plasmid pCAMBIA1305.1 was used as template DNA to amplify for 35S, NOS and a chitinase

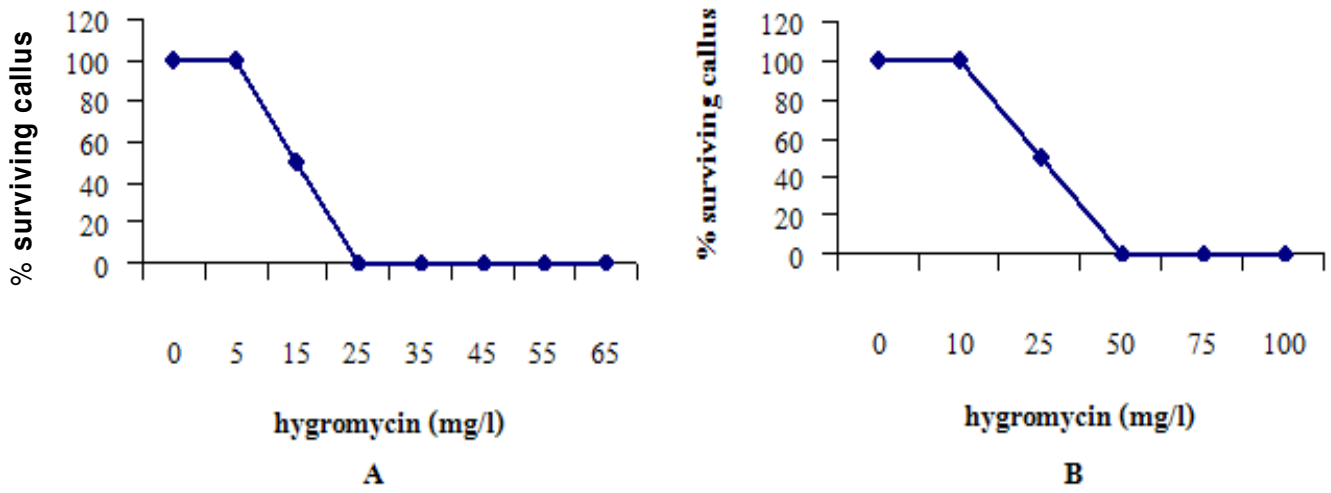

Figure 2. Percentages of viable calli under different concentrations of hygromycin: (A) callus induction; (B) regeneration.

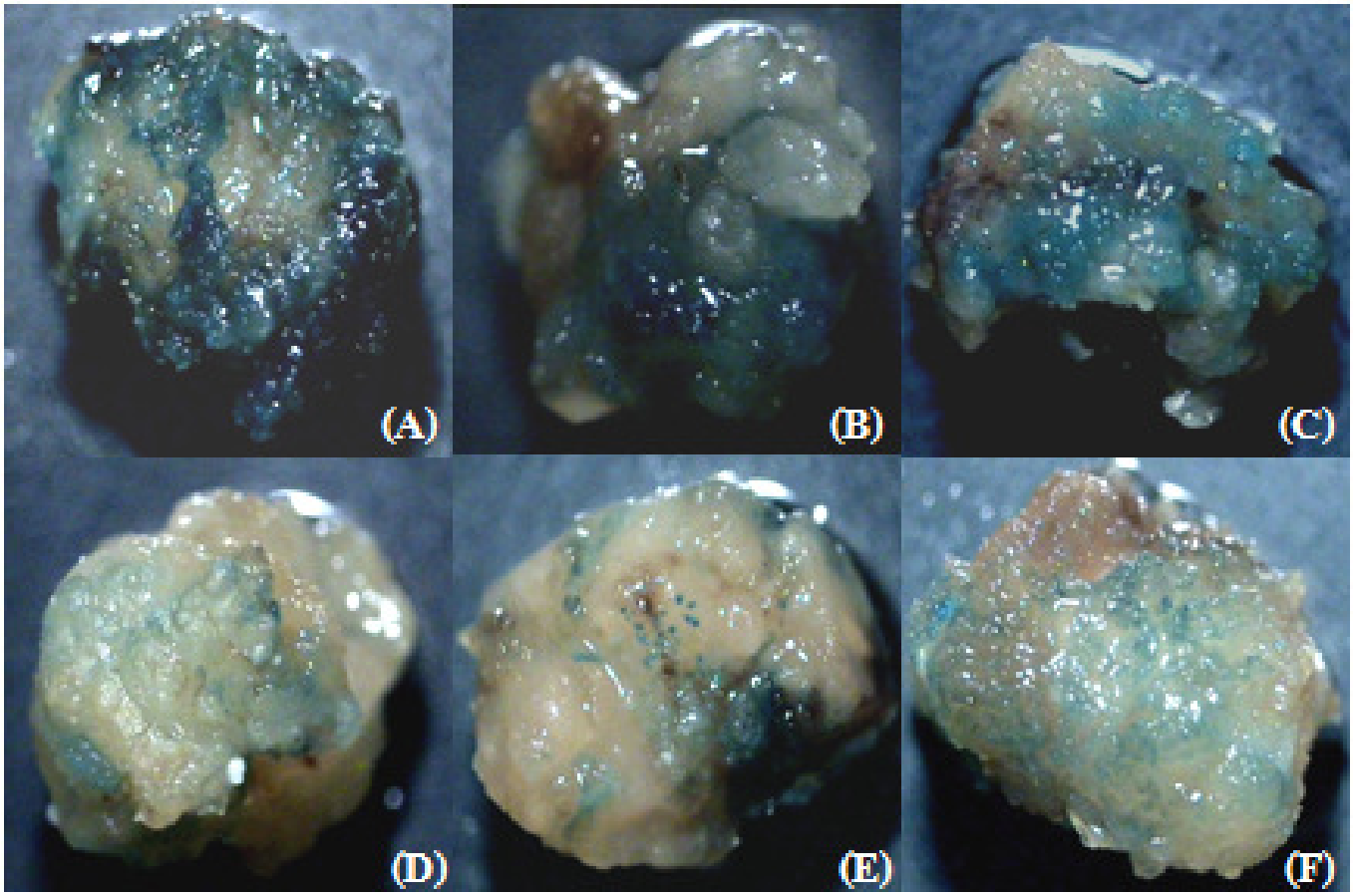

Figure 3. Blue spots represent levels of GUS activity in transformed sugarcane calli using helium pressures of was 900 (A-C) and 1,100 psi (D-F) from the stopping screen set at 9 cm.

gene. It was found that sizes of amplified fragments were 500 bp and 195 bp for 35S, 180 bp for NOS, and 464 bp for a chitinase gene; whereas, non-transformed plants did not show any expected band sizes (Figure 5). PCR technique is an additional indicator for the integration of foreign genes into plant genome (Jefferson et al., 1986;

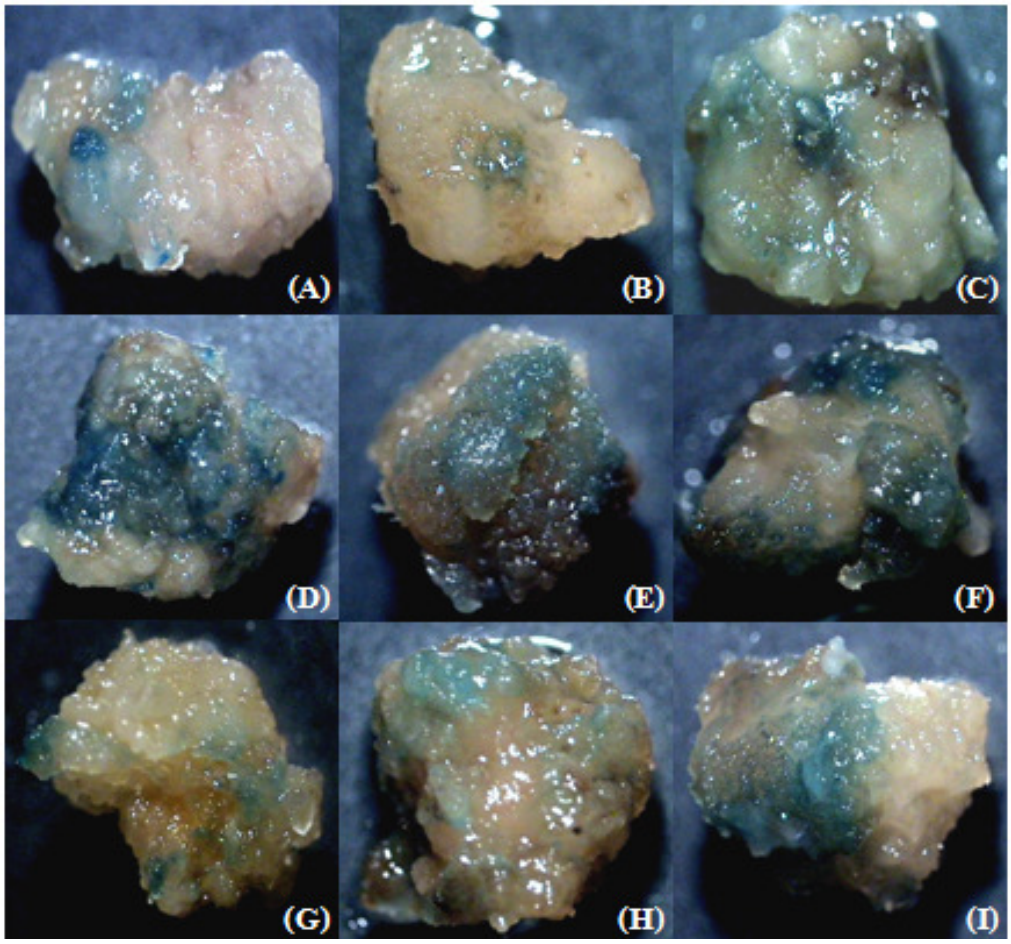

Figure 4. Blue spots represent levels of GUS activity in calli of sugarcane cv. Phil 66-07 at firing distances of 6 cm (A-C), 9 cm (D-F) and 12 cm (G-I) using helium pressures of was 900 psi

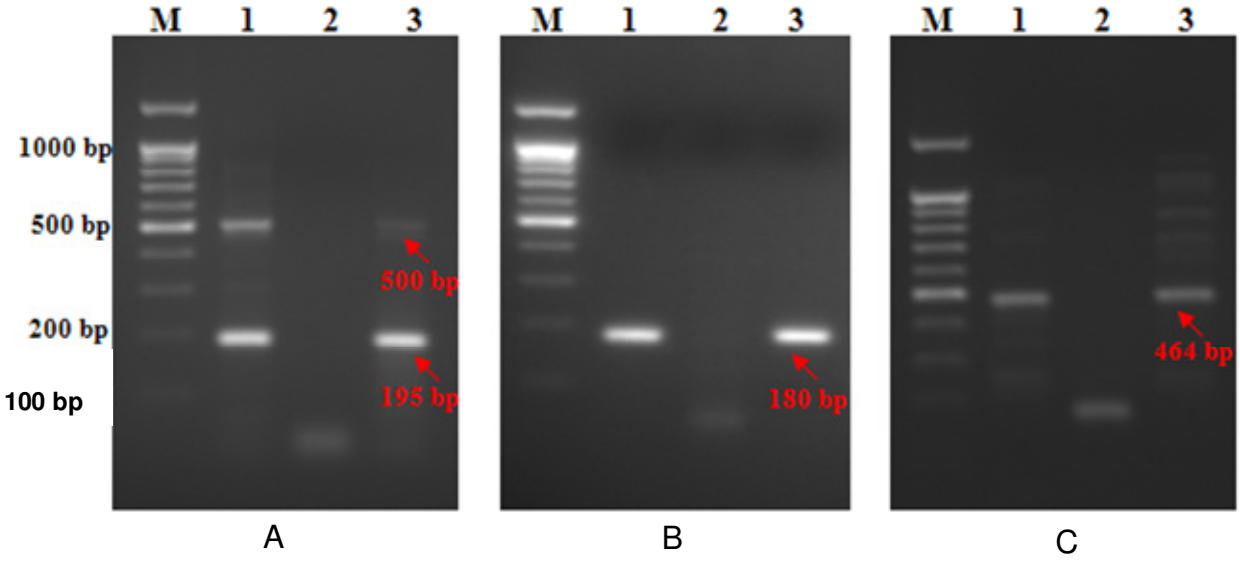

Figure 5. PCR analysis of transformed sugarcane cv. Phil 66-07 using primers to detect the 35S (A) NOS (B) and a chitinase gene (C); lane M: 100 bp DNA ladder, lane 1: pCAMBIA 1305.1, lane 2: non-transformed sugarcane, lane 3 : transformed sugarcane

Gambley et al., 1993).

Conclusions

We have successfully developed the protocol for callus induction and regeneration of sugarcane cv. Phil 66-07. Our study shows the possibility for transformation of the sugarcane with a chitinase gene via particle bombardment.

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