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Maize Genetic Transformation Procedure Improvement using *Agrobacterium tumefaciens* and FR-28 Cuban Synthetic Corn Variety as Model

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Abstract: Transgenic maize plant transformation by *Agrobacterium tumefaciens* has been widely used. However, methodology transformation efficiency is still one of the bounding aspects. In this regards, this study sought improving efficiency of transgenic maize plant transformation by *Agrobacterium tumefaciens* using as model the FR-28 Cuban synthetic maize variety. To fulfill this purpose, organoembriogenic callus lines were established from meristematic shoot and axillary buds. Next, calluses were infected with LBA4404 strain of *Agrobacterium tumefaciens* previously transformed with the pDUbar plasmid for assessing different regeneration conditions (media and osmotic treatment). As main results, the R2 treatment was the most favorable and the addition of an osmotic step, before and after infection (MInd Suc 12%), increased regeneration efficiency ($p = 0.0001$). The AAM, used during infection and modified MPC media were best combinations for obtaining a greater plant number. In detail, 35 plants per gram of calluses generated from one maize line with high regeneration capability were obtained with the improved transformation procedure. The T-DNA integration was molecularly confirmed by PCR and Southern-blot, while the bar gene expression was evidenced applying herbicide (Finale®) at 2% on the second leaf of plants recovered from genetic transformation. From this, about 33% of plants tolerated herbicide action. As conclusion, the combined application of an osmotic treatment, before and after co-cultivation and callus exposition to the light in presence of modified MPC medium during plant regeneration was the best experimental condition for increasing probability for obtaining transgenic maize plants able to tolerate different agronomic conditions such as cultivation in presence of herbicide.

Key words: *Agrobacterium tumefaciens,* maize, maize synthetic varieties, plant transformation

INTRODUCTION

Maize is one of the most important worldwide cereals (Que *et al*., 2014). At present, Cuba imports about 900,000 t, devoting more than 50% for animal feed production. In this sense, one of the strategies assessed for reaching Cuban maize self-production is the use of transgenic varieties of maize plants to effectively control plagues and in parallel decrease chemical pesticide use.

In regards to transgenic plant cultivation, it is a controversial technology but in contrast one of the most rapidly adopted in agriculture history. For instance, nowadays, 17 countries cultivate over 57 million of hectares of transgenic maize (Clive, 2013), where benefits are notable by the reached production and for avoiding pollution provoked by chemical pesticide application (Joseph *et al*., 2011).

The first transformations done for obtaining transgenic maize were achieved at the beginning of the 1990s (Gordon-Kamm *et al*., 1990). Concerning this, biobalistic and *Agrobacterium tumefaciens* bacterium transformation procedures have been applied the most for transforming this cultivar. Both methods have demonstrated high plant transformation repeatability (Gordon-Kamm *et al*., 1990; Ishida *et al*., 1996; Zhao *et al*., 2001; Frame *et al*., 2002; Tao *et al*., 2008; Mu *et al*., 2012).

Although, the number of obtained events changes depending on several factors such as transformation procedure, explant type, material genotype, regeneration capacity, plant adaptation to *ex-vitro* conditions and seed production level (Cheng *et al*., 2011).

Concerning to these limitations by factors, one of the major relies in the fact that somatic embryos (explants)

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used were generated from zygotic immature embryos of maize model lines and its derivates (Mu *et al*., 2012; Que *et al*., 2014). Where, the successful induction of somatic embryos and its final conversion to viable plants has not been an efficient routine for the majority of species. Besides, many of the few selected maize lines employed for *in vitro* culture, lacked of good agronomical quality and thus they were not representative of currently available or used as commercial lines.

On the other hand, the work with inbred lines has been approached by other strategies. Bohorova *et al*. (1995) and El-Itriby *et al*. (2003) preserved the use of immature embryos as initial explants but they supplied culture media with different carbon sources in order to increase transformant regeneration capacity. An essential requirement for transgenic plant generation is the availability of an efficient regeneration system (Oduor *et al*., 2006; Rajesh *et al*., 2008; Abrha *et al*., 2013). The employment of apical shoots has been very successful. Zhong *et al*. (1996) characterized the maize apical shoot as a structure of great morfogenic plasticity capable to generate embriogenic, organogenic and floral structures. Other authors took advantage of this quality and directly transformed these structures by using a biobalistic approach (O'Connor-Sanchez *et al*., 2002; Naqvi *et al*., 2009).

The transformation of apical shoot by *Agrobacterium tumefaciens* was described for both, hybrids and commercial varieties (Sairam *et al*., 2003; Wang *et al*., 2007). One advantage of this method is the wide host range including crops such as tobacco, maize and rice (Amin *et al*., 2004; Kutty *et al*., 2010) where agrobacterial infection is generally preferred because the resulting transgenic plants are less subject to silencing (Ali *et al*., 2007; Behrooz *et al*., 2008). Also, the *Agrobacterium tumefaciens* method would be chosen for obtaining a high number of single or low-copy transgene insertions. But, molecular and genetic basis about the interaction of the host and *Agrobacterium tumefaciens* strains remains unclear. Other limitation of the system is the strict interaction between the genotype of the plant species and the *Agrobacterium tumefaciens* strain (Ishida *et al*., 1996).

Due to these antecedents, a transformation procedure via *Agrobacterium tumefaciens*, based on the infection of calluses generated from apical shoots and axillary buds of *in vitro* germinated plantlets using the FR-28 maize synthetic variety as model was studied in this research for increasing transformation efficiency and thus obtaining a greater transgenic plant number.

MATERIALS AND METHODS

Maize plant source: FR-28 synthetic variety seeds donated by the Horticultural Investigation Institute, Liliana Dimitrova, Cuba were used as plant material to initiate callus culture.

Culture media and compositions: The composition of culture media employed were:

- **YM:** Yeast extract (0.4 g L^{-1}), mannitol (10 g L^{-1}), NaCl (0.1 g L^{-1}), $MgSO_4.7H_2O$ (0.2 g L^{-1}), $K_2HPO_4.3H_2O$ (0.5 g L^{-1}), agar (15 g L^{-1}), pH 7.0
- **MPC:** MS salts and vitamins (4.3 g L^{-1}) (Murashige and Skoog, 1962), sucrose (30 g L^{-1}), inositol (0.1 g L^{-1}), adenine (40 mg L^{-1}), casein hydrolyzate (0.5 g L^{-1}) and Gel-Rite (3 g L^{-1}), pH 5.8
- **Suc 6%:** MS salts and vitamins (4.3 g L^{-1}), sucrose (60 g L^{-1}), inositol (0.1 g L^{-1}), adenine (40 mg L^{-1}), casein hydrolyzate (0.5 g L^{-1}), cefotaxime (500 mg L^{-1}) and Gel-Rite (3 g L^{-1}), pH 5.8
- **MInd:** MS salts and vitamins (4.3 g L^{-1}), sucrose (30 g L^{-1}), inositol (0.1 g L^{-1}), adenine (40 mg L^{-1}), casein hydrolyzate (0.5 g L^{-1}) and Gel-Rite (3 g L^{-1}), acetosyryngone (100 μM), pH 5.2
- **MInd-Suc12%:** MS salts and vitamins (4.3 g L^{-1}), sucrose (120 g L^{-1}), inositol (0.1 g L^{-1}), adenine (40 mg L^{-1}), casein hydrolyzate (0.5 g L^{-1}) and Gel-Rite (3.0 g L^{-1}), acetosyryngone (100 μM), pH 5.2
- **Modified MPC:** MS salts and vitamins (4.3 g L^{-1}), sucrose (120 g L^{-1}), inositol (0.1 g L^{-1}), adenine (40 mg L^{-1}), casein hydrolyzate (0.5 g L^{-1}) and Gel-Rite (3 g L^{-1}), acetosyryngone (100 μM), pH 5.2
- **Selection MPC:** MS salts and vitamins (4.3 g L^{-1}), sucrose (30 g L^{-1}), inositol (0.1 g L^{-1}), adenine (40 mg L^{-1}), cefotaxime (500 mg L^{-1}), ammonium glufosinate (3 mg L^{-1}) and Gel-Rite (3 g L^{-1}), pH 5.8
- **MS:** MS salts and vitamins (4.3 g L^{-1}), sucrose (20 g L^{-1}), inositol (0.1 g L^{-1}) and Agar-Agar Gum (7 g L^{-1}), pH 5.6

Maize callus cultivation and maintenance: Seed disinfection process and callus induction were performed according to O'Connor-Sanchez *et al*. (2002), using MPC medium. Several callus lines were employed for assessing regeneration capacity and then the best callus lines (L1, L2 and L3) were used in subsequent regeneration condition optimization experiments.

Strain of *Agrobacterium tumefaciens* and plasmid: The pDUbar binary vector was used for transforming the LBA4404 strain of *Agrobacterium tumefaciens* (Hoekema *et al*., 1983). In this plasmid, bar gene was regulated by the ubiquitin sintetase promoter and T-nos terminator. The YM medium was used for maintaining the LBA4404-pDUbar system.

Maize callus regeneration: To optimize regeneration conditions, two treatments (R1 and R2) were tested. In both cases, 3 g of calluses of each maize line (L1, L2 and L3) were used:

- **R1 treatment:** Calluses were plated in MPC medium in darkness for two weeks. Later in Suc 6% medium for one week with a photoperiod of 16 h light and finally were sub-cultivated in MS medium
- **R2 treatment:** It was similar to R1 treatment, including a subculture in MPC medium (with photoperiod 16 h light one week) before the culture in Suc 6% medium. All experiments were always performed at 27°C

Maize callus transformation: In transformation experiments, the influence of an osmotic treatment previous and after the infection with *Agrobacterium tumefaciens* was studied. Besides, two infection media MS and AAM (Hiei *et al*., 1994) and two co-culture media MPC and modified MPC were also analyzed. For the osmotic treatment assessment, 4 h before callus transformation, calluses were divided in two groups and similar amounts were placed on assessed media MInd and MInd-Suc12%. After infection, calluses were kept under same osmotic conditions for 16 h at 22°C in darkness. Then, LBA4404-pDUbar *Agrobacterium tumefaciens* was inoculated into liquid YM medium supplemented with 100 mg L^{-1} spectinomycin, 100 mg L^{-1} streptomycin and 50 mg L^{-1} rifampicin and incubated at 28°C overnight. Next, bacterium was re-inoculated in AIB medium (Arias and Sarria, 1995) for 24 h. Subsequently, culture was centrifuged and resuspended in MS or AAM (infection media), adjusting infection culture density to $3\text{-}5\times10^{9}$ cell mL^{-1}. In parallel, plant material was prepared seven days before transformation. Four grams of L3 line organoembriogenic calluses (previously selected in the regeneration assay) were sub-cultivated in MPC medium, before osmotic treatment. In infection experiments, calluses (0.5 g) were submerged in 5 mL of MS or AAM for 10 min. Calluses were incubated in osmotic conditions as it was previously described. After this time period, calluses were transferred to co-culture media: MPC and modified MPC at 22°C for 72 h, in darkness conditions. Finally, callus co-cultures of different treatments were washed with sterile distilled water and cultivated in MPC medium supplemented with cefotaxime 500 mg L^{-1} for one week. Subculture selection was performed once every two weeks in MPC. Groups of calluses that proliferated during these conditions were regenerated using the R2 treatment previously described.

Ammonium glufosinate tolerant transgenic plant generation: A final transformation experiment using four grams of L3 maize line calluses was carried out. Best treatment combinations selected from previous tests were applied. The generated plants were then transferred at a plant density equal to 30 plants per centimeter to greenhouse conditions.

Transformant molecular analysis: Total DNA was isolated from 0.5 g of leaves of seven regenerated plants following Shure *et al*. (1983) method. The same samples were used for the PCR and Southern blot assays. The PCR primers used for a fragment of 402 pb of bar gene were P1: 5'CGAGACAAGCACGGTCAACTTC3' and P2: 5'AAACCCACGTCATGCCAGTTC3'. The PCR program was as follow, denaturalization cycle at 94°C for 5 min, followed by 40 cycles of 1 min at 95°C, 2 min at 62°C and 2 min at 72°C. It was followed by a final extension at 72°C for 10 min. PCR's products were separated by electrophoresis in agarose at 1% (w/v) and stained with ethidium bromide. The gel was visualized and photographed by light UV. In the southern blot assay, 10 μg of total DNA of each sample were employed in the hybridization step. For this purpose, DNA was digested with the Hind III enzyme (only one recognition site in the T-DNA inserted fragment). Digestion products were separated by agarose electrophoresis in 1% (w/v) gel and transferred into Hybond N+nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The DNA was hybridized with P^{32}-labeled bar probe (Prime-a-Labeling System, Promega, Madison, USA), during 12 h at 65°C. Ten micrograms of the pDUbar plasmid served as positive control. The analysis was performed following the standard procedure described by Sambrook and Russell (2001) and the membrane was placed in X-ray (AGFA, Japan) and stored at -70°C for five days for result visualization.

Finale® herbicide application: After two weeks of transplant, Phosphinotricim Acetyl Transferase (PAT) activity was measured by applying a solution containing 2% of Finale® on the transgenic plants and controls. Two applications were done on the third part of the leaf of each plant. In this sense, the first application was done when plants reached a 15 cm-height approximately (in the fourth leaf), whereas the second one was done 10 days later. The evaluation was performed one week after second application.

Statistical analysis: A two ways-ANOVA and Bonferroni post-tests were performed to compare the means of frequency regeneration of each line (L1, L2 and L3) in multiplication stage in MPC medium and light effect, before osmotic treatment during regeneration assay, using 95% as confidence interval. The number of independent experiments were always three. One way ANOVA was performed to compare the means of plant regeneration frequency after different *Agrobacterium tumefaciens* transformation processes and the Tukey's Multiple Comparison test was used to discriminate among variables with statistical differences. In both tests the statistical confidence interval was 95%.

RESULTS AND DISCUSSION

Maize line selection and regeneration setting optimization: FR-28 is a synthetic variety of maize generated by the combination of several germoplasmas. It is an adaptable plant to different culture conditions and has a high level of resistance to tropical diseases (Rabi *et al*., 1997). In this study, the protocol described by O'Connor-Sanchez *et al*. (2002) for establishing organoembriogenic maize calluses was used for maize line selection and regeneration setting optimization.

As it was illustrated in Fig. 1a, organogenic structures were observed in seed germinated sections after three weeks of callus cultivation in MPC medium. As general characteristics, these calluses were developed in a major percentage from the apical shoot and in minor degree from axillary buds. In addition, the callus culture in MPC medium also stimulated the somatic embryos development in the darkness, stage in which a high rate of multiplication was observed. Besides, organogenic structures were detected in fewer quantities (Fig. 1b). The establishment of these structures has been described as organoembriogenic calluses (O'Connor-Sanchez *et al*., 2002).

Calluses were generated from 480 maize seeds and distributed in six individual experiments. The initial regeneration experiment results are shown in Table 1. In this sense, about 28.5±9.7% of the established lines were able to regenerate plants. The variability of regeneration capability could be explained by the own characteristics of this synthetic variety. Compared with model lines, this synthetic variety has a low regeneration capability. Regarding this, other authors have also demonstrated that several factors can affect the plant regeneration capability such as the explants type, genetic background, embryo size and culture medium (El-Itriby *et al*., 2003). In the case of the FR-28, it is not clear yet the influence of these factors on plant regeneration capability. However, the percentage of seeds that developed calluses (88.95%) was higher than that those previously reported by O'Connor-Sanchez *et al*. (2002) using the same variety (up to 75%).

One of the most important requirements for the plant genetic transformation is to dispose of a regeneration efficient protocol. Herein, regeneration conditions were optimized using three maize callus lines (L1, L2 and L3), which were divided in fractions with similar weights to compare each treatment (R1 and R2). In this stage, differences in the behavior of the callus groups cultivated under different culture conditions were detected. The multiplication stage of the maize calluses in MPC with a photoperiod of 16 h light before the osmotic treatment significantly stimulated the callus regeneration in the three studied lines. Taking into consideration the number

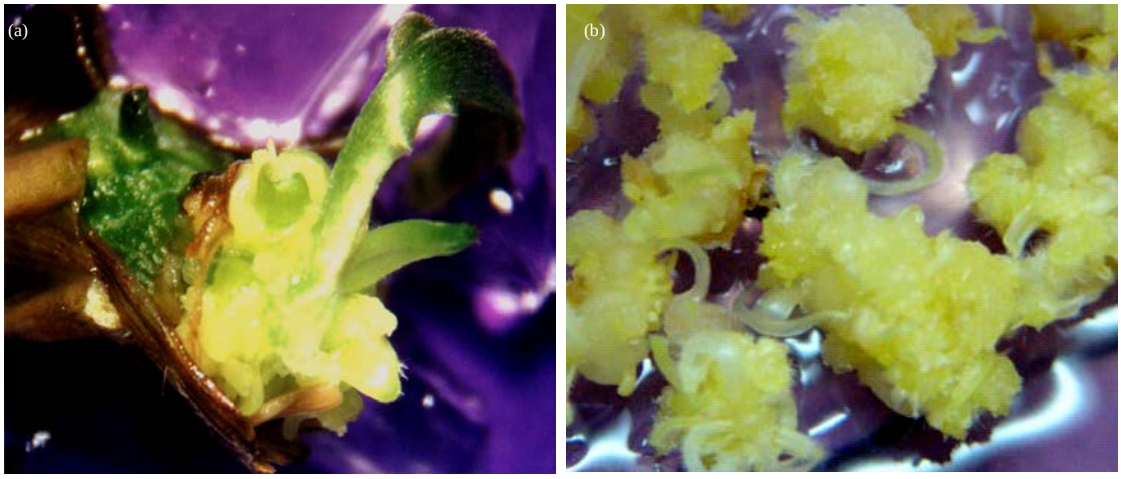

Fig. 1(a-b): Induction and multiplication of maize calluses from the FR-28 synthetic variety, (a) Primary calluses derived from the apical shoot and/or axillary buds and (b) Organoembriogenic calluses multiplication in the dark

Table 1: Callus regeneration efficiency from seeds of the variety FR-28

Individual experiments	1	2	3	4	5	6	Mean	SD
Analyzed seeds	80.00	80.00	80.00	80.00	80.00	80.00	80.0	-
Seeds that developed organoembriogenic calluses	73.00	72.00	69.00	74.00	67.00	72.00	71.2	2.6
Regenerated lines	19.00	15.00	12.00	25.00	19.00	32.00	20.3	7.2
Regeneration efficiency (%)	26.03	20.83	17.39	33.78	28.36	44.44	28.5	9.7

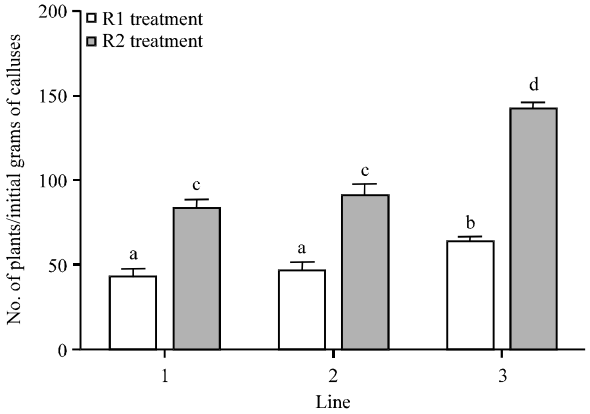

Fig. 2: Regeneration of organoembriogenic calluses of the FR-28 variety. Calluses were transferred to different culture media in the subsequent order for inducing regeneration. R1 treatment: MPC medium in darkness, Suc 6% and MS medium in light (black bars). R2 treatment: MPC medium in darkness, MPC medium one week in light, Suc 6% and MS medium in light conditions (bars with diagonal lines)

Fig. 3: Illustration of the development of embryos from organoembriogenic calluses in the maturation medium

of plants obtaining in all tested lines (L1 (82.7±5.9), L2 (90±6.4), L3 (141.3±4.3)), R2 treatment was the most efficient ($p = 0.0001$). The L3 line behaved better in both regeneration conditions ($p = 0.0010$). The frequency of regeneration of L3 was higher for the R2 treatment, for an average of 142 plants per gram of callus sub-cultivated in the darkness. Results obtained for L1 and L2 lines in the R2 treatment were 82 and 91 plants per gram of calluses sub-cultivated in the darkness as average, respectively, which were similar to those obtaining for O'Connor-Sanchez *et al.* (2002) using the same variety but R1 treatment (Fig. 2).

Calluses cultivating in the maturation medium (Suc 6%) during a week generated seedlings that were then transferred to the greenhouse. Function of sucrose in the culture medium was as carbon source and as osmotic regulator, being both functions important for plant development. The culture of calluses in this cultivation medium promoted maturation of a great number of somatic embryos and increased plant regeneration probability (Fig. 3). In some cultures, the increase in the endogenous abscisic acid level has been determined in the tissues cultivated under this experimental treatment. This hormone may induce growth of the root and stem in osmotic stress conditions (Huang *et al.*, 2012). Several tissue culture works performed in maize and wheat recommend the use of an osmotic treatment during plant regeneration to stimulate embryo scutellum development to detonate the conversion to plant (Pereira *et al.*, 2008; Raja *et al.*, 2010).

Transformation condition optimization: In order to optimize transformation conditions, the L3 callus line was choose to be transfected with LBA4404 strain previously transformed with the pDUbar construction to study the influence of the osmotic treatment before and after the infection, the effect of two infection broth (AAM and MS) and different co-cultivation conditions. The combination of osmotic treatment before the infection with *Agrobacterium* in AAM broth, the co-culture of calluses in MInd-Suc12% and modified MPC was the best treatment for increasing the number of plants recovered (32 plants per gram of calluses sub-cultivated in the darkness, $p = 0.0001$) (Table 2). To reach the best condition for the transformation of vegetable tissue, a high concentration of sucrose before and during the co-cultured period was used. In this concern, several authors have used similar conditions of osmotic stress but in biobalistic research in maize and soybean (O'Connor-Sanchez *et al.*, 2002; Naqvi *et al.*, 2009).

Table 2: Number of transformed plants obtained in different treatments

Infection media	Osmotic treatment (4 h before infection and 16 h in co-culture)	Co-clture (72 h)	No. of plants
MS	Mind	MCP	10[a]
		Modified MPC	20[c]
	Mind Suc 12%	MCP	8[a]
		Modified MPC	26[d]
AAM	Mind	MCP	15[b]
		Modified MPC	28[d]
	Mind Suc 12%	MCP	15[b]
		Modified MPC	32[e]

Different letters are No. of plants show statistical differences among them ($\alpha = 0.05$)

On the other hand, Zhao *et al*. (2001) and Frame *et al*. (2002) described the favorable effects of osmotic treatment in maize immature zygotic embryos before the transformation with *Agrobacterium*. Uze *et al*. (1997) obtained an increase in the efficiency of transformation of immature embryos of rice on having provoked the plasmolysis of the tissues (sucrose 10%). In another study, Trifonova *et al*. (2001) reported significant improvements in the transformation of barley on having added 10% of sucrose to the culture broth 5 h previously to the infection and later, during the first 18 h of co-culture.

The composition of different infection media was another aspect taken into consideration. The AAM medium was firstly used in rice transformation (Hiei *et al*., 1994) and later, with some modifications, in cultures like maize and vetiver (Ishida *et al*., 1996; Frame *et al*., 2002; Yang *et al*., 2006). In these protocols the employment of AAM medium increased the number of plants obtained in all analyzed variants. In this sense, arginine and aspartic acid have been aminoacids previously used in works on tissues culture and constitute important sources of reduced nitrogen. The myo-inositol is also important for plant normal growth and development because it participates in auxin storage and transport, cell wall formation among other important processes of the plant metabolism (Loewus and Murthy, 2000).

The utilization of the modified MPC medium in the first stage of co-culture, increased the frequency of plants obtained during the transformation. The addition of sucrose, glucose and acetosyringone to the induction and co-culture medium play a significant role in the monocotyledon plant transformation such rice, maize and wheat (Hiei *et al*., 1994; Ishida *et al*., 1996; Cheng *et al*., 2004; Rashid *et al*., 2011). It has been shown the involvement of these compounds in the proliferation of plant material. Tzfira *et al*. (2002) emphasized the function of monosaccharides and acetosyringone in the interaction plant cell-Agrobacterium. These compounds interact with the recipients that unleash the expression of the genes vir.

Generation of transgenic plants tolerant to ammonium glufosinate: Once regeneration and transformation conditions were standardized, a new transformation was done using the same system (LBA4404 pDUbar). Calluses tolerant to ammonium glufosinate were observed after four weeks of the selection in the darkness (Fig. 4a-c). A higher rate of calluses multiplication and more brilliant appearance was observed in plates with putative transformed callulses. The non-transformed calluses (negative control) cultivated under the same selection conditions had a dark color or appearance (died) due to accumulation of ammonium and decrease in the concentration of glutamine other amino acids (Steckel *et al*., 1997). When callus selection period concluded, calluses were then transferred into MPC medium without ammonium glufosinate with a photoperiod 16 h light: 8 h dark. Under these conditions, the ammonium accumulated in tissues is cytotoxic, because it affects the electron transport chain in cells exposed to the light, (D'Halluin *et al*., 1992). Summarizing the non-transformed calluses died under these incubation conditions while putative transformed calluses developed organogenics structrures (Fig. 4d, e). Seedlings obtained from the maturation medium (Suc 6%) were next transferred to the MS medium for producing roots and to complete the seedling development (Fig. 4f). After 3 months, 140 plants were obtained and cultivated under confined conditions, of which 126 (94%) reached the maturity (T0 generation). In this assay about 35 plants by gram of transformed calluses as average were obtained. This result was higher than those obtained by O'Connor-Sanchez *et al*. (2002) using the same variety but transformed by mean of a biobalistic procedure. Shou *et al*. (2004) had also a lower transformation frequency using the result of the number of rearrangements that happened during the transformation process when a biobalistic procedure was used in another plant variety. However, Dai *et al*. (2001) obtained similar results comparing these two transformation methods in rice. The obtained plants were kept in a greenhouse up to the seed cropping. In addition, no morphologic differences were detected among regeneration controls and putative transformed or transgenic plants.

Integration and expression of the bar gene in putative transgenic clones: To confirm the insertion of the transformation cassette in the transformed plants, a PCR

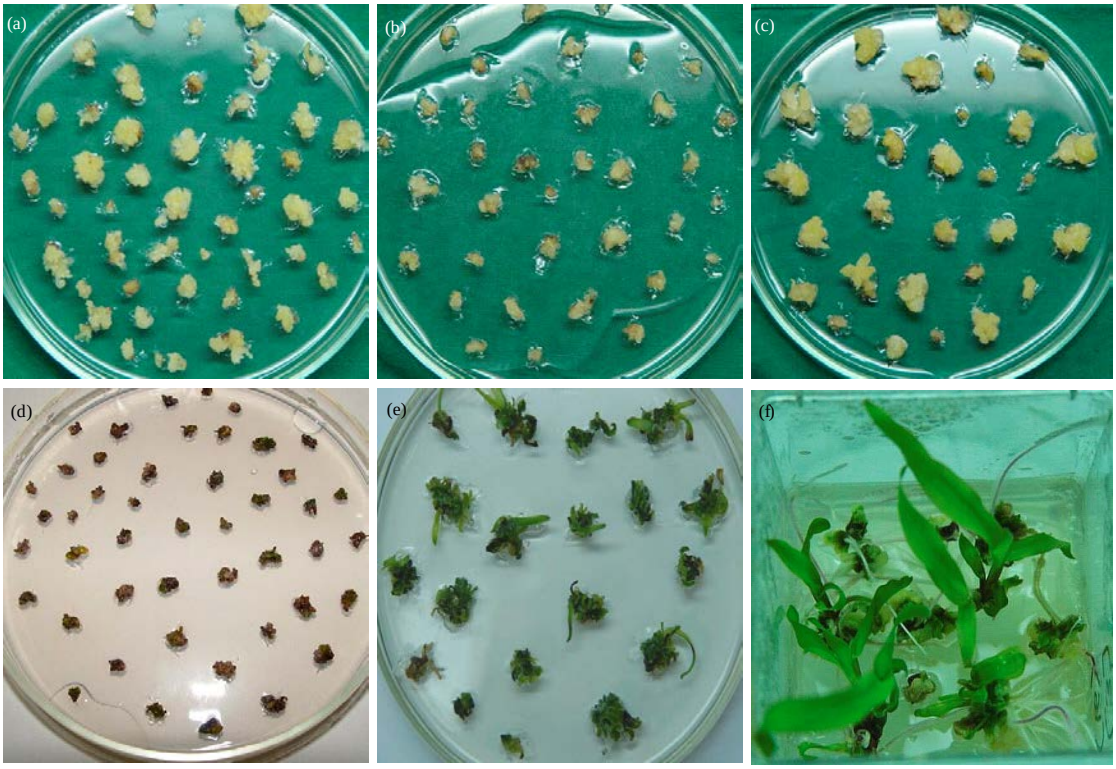

Fig. 4(a-f): Maize transgenic plant generation with the LBA4404 pDUbar system, (a-c) Organoembriogenic calluses of maize after four weeks in selection MPC medium; (a) Non-transformed calluses in selection MPC medium without ammonium glufosinate, (b) Non-transformed calluses, (c) Transformed calluses, (d-e) Callus response in the light and R2 regeneration process, (d) Non-transformed calluses, (e) Seedlings generated from transformed calluses and (f) Rooted plants in MS medium

amplification and Southern blot assay for a bar gene fragment were performed. With this purpose, total genomic DNA was extracted from a leaf of six transgenic plants. All analyzed samples had the transformation cassette (Fig. 5a). In addition, the Southern blot performed with 10 μg of total genomic DNA of each plant previously digested with HindIII and hybridized with bar probe showed the integration of the transformation cassette with the bar gene in all analyzed samples of transgenic plant (Fig. 5b). Each band observed in the hybridization analysis was considered as an insertion copy of the transgene in the genome of the synthetic maize variety. In detail, five of six analyzed transgenic plants showed a gene single copy (Fig. 5b: Lines 2-5 and 7) whereas one sample had two integration sites (Fig. 5b: Line 6). Results indicated that at least three hybridization patterns (Pattern 1: Plants 2 and 5; Pattern 2: Plants 3, 4 and 7; Pattern 3: Plant 6). Plant 6 exhibited a combination of both patterns. The low transgene copy number is typical of transformations via *Agrobacterium tumefaciens*, which is a guarantee of stability of the integrated fragment (Ishida *et al*., 1996).

Functional PAT activity evaluation: In this study, the detoxifying PAT activity was studied in T0 generation cultivated under greenhouse conditions described by Gordon-Kamm *et al*. (1990). The fourth leaf of healthy plants was painted with a Finale® 2% solution. All plants were analyzed and showed different tolerance levels to the herbicide. As result of the painting procedure, 43 of 132 plants resisted herbicide application, which corresponded with 33% of tolerance (Fig. 6). All non-transformed plants died after the first herbicide application. Similar results were obtained by Yang *et al*. (2006) during embriogenic callus transformation with surfactants and LBA4404-*Agrobacterium tumefaciens* strain. Conversley, Ombori *et al*. (2013) showed a less transformation efficiency of calluses (0-8.7%) using immature zygotic embryos of tropical maize lines with different *Agrobacterium tumefaciens* strains and

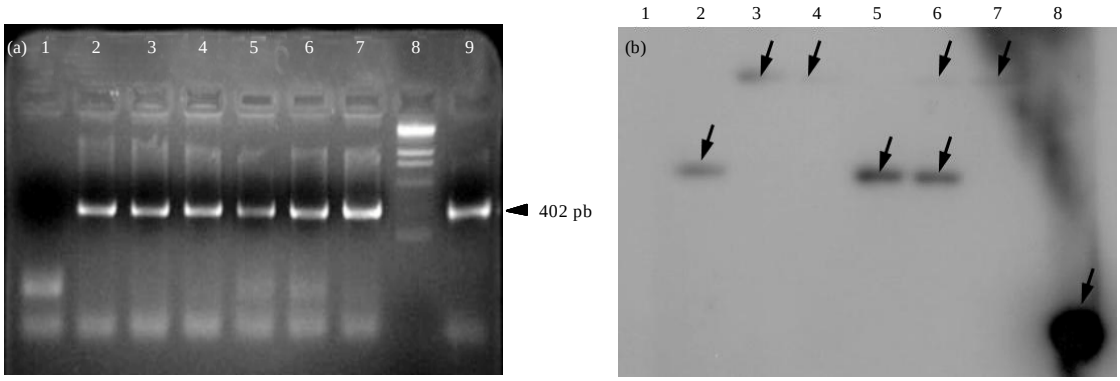

Fig. 5(a-b): Illustration of the PCR and Southern blot for bar gene fragment in six transgenic plants T0. In (a) Line 1: DNA from non-transformed plant (negative control), Lines 2-7: Transgenic plants, Line 8: Molecular weight marker 1 kb Promega, Line 9: pDUbar plasmid (positive control). In (b) Southern blot analysis of six transgenic plants to determine the bar gene copy number (10 μg of total genomic DNA were digested with HindIII and hybridized with P32-labeled bar probe), Line 1: Non-transformed plant, Lines 2-7: pDUbar transgenic plants, Lane 8: pDUbar plasmid (positive control)

Fig. 6(a-c): Results of the functional PAT activity evaluation in pDUbar plants cultivated under greenhouse conditions. (a) Non-transformed plants without Finale® 2% application, (b) Non-transgenic plants after Finale® 2% application and (c) Transgenic plants (pDUbar plasmid) tolerant to Finale® 2%

increasing the acetosyringone concentration up to 200 μM during the infection and co-culture.

CONCLUSION

The combined application of an osmotic treatment before and after co-cultivation and callus exposition to the light in presence of modified MPC medium during plant regeneration was the best experimental condition for increasing probability for obtaining transgenic maize plants able to tolerate different agronomic conditions such as cultivation in presence of Finale® herbicide.

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