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An efficient regeneration and genetic transformation of maize through *Agrobacterium* and particle bombardment in immature embryos

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ABSTRACT

An efficient *Agrobacterium* mediated transformation system, from which transgenic tropical maize plants was directly generated without previous crosses with laboratory or temperate lines was established in present study. Experimental evaluations are focused on two main issues: i) Establishment of appropriate tissue culture conditions, which induced somatic embryogenesis from the immature embryo cells, and ii) The delivery of T-DNA toward these cells. A high rate of embryogenic calli, generated from immature embryo was obtained when 2-4 D ($4mgL^{-1}$) was added to the MS based induction medium. Regeneration rate was observed upto 19 plants per gram in the calli by using BAP and KIN at $0.5mgL^{-1}$. Regarding the *Agrobacterium* strains evaluated for their transformation capability on the tropical maize line MU-2092 used, best results are obtained from the strain when applied at OD 550_{nm} = 0.5-1.0. Physical micro-wounds before the Agro-infection proved to be an excellent way to promote both the T-DNA transfer toward the immature embryo and the acceleration in rates of transient GUS expression when compared with particle bombardment. The highest frequencies of transient GUS expression corresponding to the embryos as well as the regeneration of whole transgenic plants emerged from them, was obtained using immature embryos wounded by bombarding at 80 lb/in^2. Analysis of the progenies confirmed the sexual transmission of the introduced genes and their stable expression.

Key words: Agro-infection, Immature embryos, Somatic-embryogenesis.

Abbreviations: 2-4 Dichlorophenoxy Acetic Acid (2-4 D), 6-Benzylamino Purine (BAP), Indole Butyric Acid (IBA), Naphthalene Acetic Acid (NAA).

INTRODUCTION

Maize is one of the most important crops around the world because of its importance as food and feed in the past and present. So, tissue culture approaches to biotechnology, in order to incorporate desirable traits on several maize lines (Armstrong 1985). Among the biotechnology tools, those related to transferring DNA have received special attention, resulting in several strategies such as biolistic or *Agrobacterium* mediated genetic transformation. A lot of advantages have been associated with the *Agrobacterium* mediated transformation (Zhao *et al*., 2001) by the possibility of transferring large DNA segment into recipient cells, the generation of a high number of stable, correctly expressing transgenic events, while producing less transgene rearrangements. Additionally, this represents a simple technology with low cost. In light of the above mentioned advantages, several studies related to the *Agrobacterium* mediated transformation of maize have focused in diverse factors associated with this system, such as the plant genotype and developmental stages of the explants, strains and cell bacterial density by Akula *et al*., (1999), in addition to phenolic compounds, co-cultivation, vectors, pH, temperature as well as the composition of medium. At first, this parameters tried only on cotyledons by Beyer *et al.*, (1976) using the soil phytopathogen *Agrobacterium tumefaciens*. Initially, it was not clear if this technology extended to monocotyledons, as they are not natural host to *Agrobacterium tumefaciens*. However an efficient method of transforming rice by *Agrobacterium* was reported by Cho *et al*., (1998) concluded that it can be transform to monocot. Later, this was extended to transform the cereals such as maize, wheat, barely and sorghum by *Agrobacterium* mediated transformation and now it is highly recommended for maize varieties with tissue culture.

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For the successful production of transgenic plants in any species, foreign genes must be delivered to undifferentiated, dedifferentiated or dedifferentiating cells that are actively dividing or about to divide and that are capable of regenerating plants. In maize, the material of choice is immature embryos and all protocols mediated by particle bombardment or *Agrobacterium tumefaciens* for efficient production of transgenic maize have solely employed in immature embryos. Thus, the primary determinant of a successful transformation by tissue culture response of immature embryos was supported by Frame *et al*., (2006). The types of cells that grow from immature embryos and subsequent characteristics in growth and regeneration make successful transformation.

MATERIALS AND METHODS

The present investigation was carried out at the Crop Physiology Lab in Acharya N.G Ranga Agricultural University, Naira in 2015. MU-2092 is identified as a genotype susceptible to agro-infection and capable of producing regenerative type-II calli has been conducted. An immature zygotic embryo (1.5-2.0mm) was aseptically dissected from green house-grown ears harvested 12-15 days after pollination. For callus induction and maintenance, an immature embryo was cultured oriented with the embryo-axis side in contact with the medium. The frequency of embryogenic callus (Rueb *et al*., 1994) formation varied depending on the combinations of applied plant growth regulators. On the sub-culture medium free of plant growth regulator, the primary calli turned brown, watery and died. 2, 4-D at 4mgL^{-1} alone stimulated the formation of embryogenic callus. For regeneration, inoculated explant was incubated in a growth chamber at 26^{0}C with a photoperiod 16:8 (light: dark). After 3weeks, embryogenic callus produced. This embryogenic tissue was sub-cultured to fresh medium for every 20 days. Regeneration capacity of the different induced calli was evaluated for three different regeneration media, all of them based on basal MS medium (Murashige and Skoog 1962). MS_1 hormone free and 30gL^{-1} sucrose (dead callus): MS_2 hormone free 60gL^{-1} sucrose and 100mgL^{-1} myo-inositol (callus) and MS_3, 30gL^{-1} sucrose, 0.5 mgL^{-1} KIN & 0.5mgL^{-1} BAP, 200mgL^{-1} casein hydrolysate and 2mgL^{-1} proline. The P^H of all media was adjusted to 5.8 and adds 8gL^{-1} Agar.

RESULTS AND DISCUSSION

Callus induction: Approximately 80% of the immature embryos grown on medium containing $AgNO_3$ are able to generate calli, compared to 58.2% of those induced in absence of $AgNO_3$. The total rate of embryos producing callus increased 20–25% by the addition of silver nitrate to the induction medium, 15 mgL^{-1} seemed to be the optimal level for the induction of friable embryogenic type-II callus. Additionally, increased somatic embryogenesis by the addition of $AgNO_3$ has been reported in different cultures such as wheat (Wu *et al*., 2006), coffee (Fuentes *et al*., 2000) and L. barbarum (Li *et al*., 2001). This tendency has been reported by Huang and Wei (2005), when low concentrations of $AgNO_3$ significantly increased the production of embryogenic callus in sub culturing medium, but concentration to 20mgL^{-1} inhibited it strongly. Thus, determining the minimal amount of Ag+ necessary for inducing this embryogenic response is essential.

Plant regeneration: Different regeneration media was initially evaluated, best results are found with MS_3 medium (Bohorova *et al*., 1995); thus, it was selected for posterior plant regeneration. A significant increase in regeneration rates was found when calli are induced under presence of $AgNO_3$ and with hormonal concentration BAP and KIN 0.5mgL^{-1}. Plant regeneration has shown below (Figure 1).

Only 4.4 plants per g fresh weight (FW) of calli induced from control medium, were as regenerated medium upto 12.3 and 19.1 plants per g fresh weight (FW) of calli induced on the presence of hormones BAP and KIN 0.5mgL^{-1} and 15mgL^{-1} $AgNO_3$ are regenerated. It is well known that $AgNO_3$ influences cell division, Cyto-differentiation, and regeneration capacity (Songstad *et al*., 1991). At present work, the capacity to form embryogenic calli was correlated with the efficiency to regenerate plantlets (Table 1).

Similar results are reported by Carvalho *et al*. (1997) when testing the regeneration ability of embryogenic calli induced in several maize inbred lines; the highest number of regenerated plants/g calli in each MU-2092 are observed in medium containing 15 mg L^{-1} AgNO3 and hormones BAP (0.5 mg L^{-1}) and KIN(0.5 mg L^{-1}). When multiple shoots grown on regeneration medium was divided and transferred to rooting medium containing 1.0 mgL^{-1} IBA, thick white roots developed in about 2 weeks. Plantlets with well-developed roots are transferred to soil and transplanted into greenhouse. Nearly all of the established plants are morphologically normal, similar to the original lines, and exhibiting male and female fertility. Using this optimized protocol, plantlets was regenerated from mature and immature embryos. The frequency of plant regeneration was ranged from 23.44% to 87.5 % (Table 2)

Plasmid construction: The *Agrobacterium* strain LBA4404 and plasmid p^{CAMBIA} 1301 was obtained from Biotechnology

Table 1: Effect of various concentrations of growth hormones with respect to shoot regeneration from immature embryos explant in maize.

Growth regulators (mgL^{-1})	Responding explant	Regeneration frequency	Number of shoots per explant
0.2 BAP+0.5 KIN	70±5.77	23.44±5.77	1.3±0.67
0.4 BAP+0.5 KIN	70±5.77	53.3±3.33	1.3±0.33
0.5 BAP+0.5 KIN	90±6.77	87.5±3.33	4.2±0.33
0.6 BAP+0.5 KIN	80±6.77	76.7±3.33	2.7±0.33

Table 2 : Effect of Indole butyric acid on percentage rooting

IBA	No of explants	Percent of rooting
0.2	20	20
0.4	20	35
0.6	20	43
0.8	20	50
1.0	20	80
1.2	20	63
1.4	20	Callus

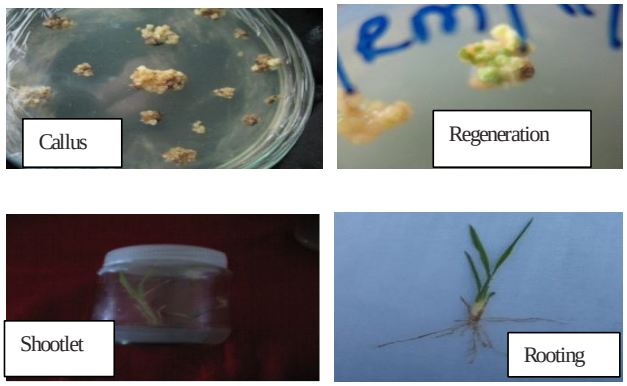


Fig 1: Effect of growth hormones on shoot regeneration from mature and immature embryos

and Molecular Genetic Lab, Department Of Botany, Osmania University, Hyderabad. Strain LBA4404 of *Agrobacterium tumefaciens*, all containing standard vector p^{CAMBIA} 1301 was tested in their ability to infect immature embryos of maize (Miller *et al.*, 2002). The T-DNA region of this plasmid contains the reporter gene Gus and the selected marker gene, both under the control of CaMV 35S promoter (Figure 3). Moreover the Gus gene contained a portable intron (GUS) to prevent GUS activity coming from *Agrobacterium tumefaciens* cells.

***Agrobacterium* transformation:** Infection, co-cultivation and resting media used in the present work contains MS salts and vitamins 1.5mgL^{-1}, 2-4 D and 0.7gL^{-1} proline and addition of the following components (Dahleen *et al.*, 2002). Infection medium contained 30mgL^{-1} sucrose and supplemented with 100µm Acetosyringone (sigma, st.louis) before use. Co-cultivation medium contains 30gL^{-1} sucrose, 350mgL^{-1} L-Cysteine, 0.85mgL^{-1} silver nitrate, 100µm AS and 8gL^{-1} Agar (pH 5.8). Resting medium contained 30gL^{-1} sucrose, 0.5gL^{-1} MES, 0.85mgL^{-1} silver nitrate, 250mgL^{-1} cefotaxime and 8gL^{-1} purified agar (pH 5.8). Selection was applied from callus to transgenic plants supplemented with kanamycin 50mgL^{-1} I, Kanamycin 250 mgL^{-1} II to Kanamycin 500mgL^{-1} III.

For the infection, *Agrobacterium tumefaciens* cultures was grown for 2-4 days at 20^{0}C on YEP medium (5gL^{-1} Yeast extract, 10gL^{-1} Peptone, 5gL^{-1} Nacl, 15gL^{-1} Agar,

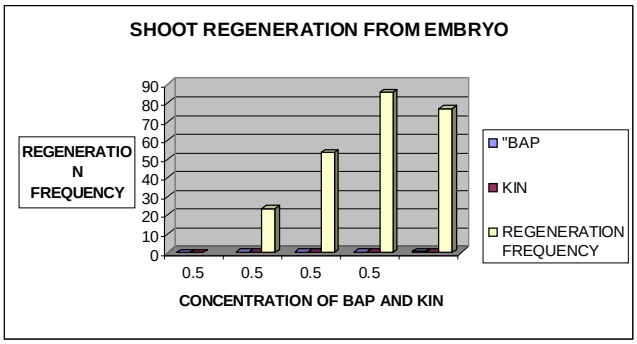


Fig 2: Graphical representation of growth hormones on shoot regeneration from immature embryos in maize.

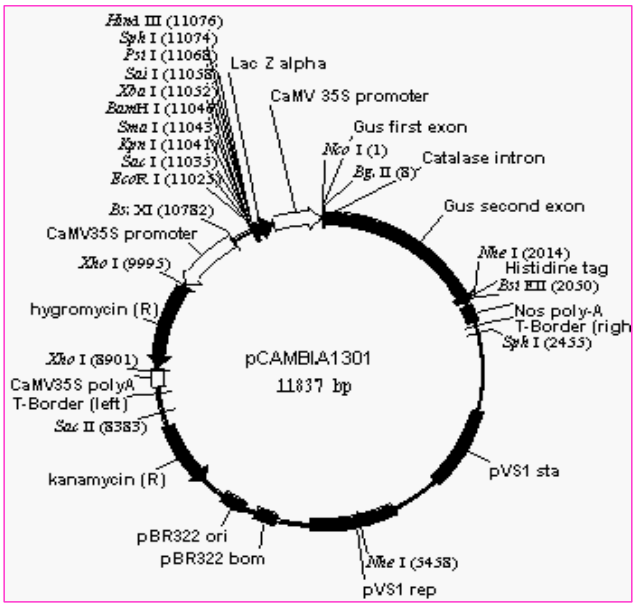


Fig 3: Systematic representation of T DNA region of the standard binary transformation vector p^{CAMBIA}1301. Gus-exon, β-glucuronidase gene contains exon; CaMV35S promoter; CaMV35S Poly A terminator; Hygromycin as a selection marker.

pH 6.8 amended with 50mgL^{-1} kanamycin (for transformation vector) and 0.5mgL^{-1} Rifamcin. Prior to infection, one full loop (3mm) of bacterial culture was scraped and suspended in infection medium (30gL^{-1}sucrose, pH4.2) and supplemented with 100µm Acetosyringe. Bacterial suspension of 5ml was measured and adjusted to three different optical densities (O.D) 0.1-0.5, 0.5-1.0 and 1.0-1.5. The dissection of immature embryos from the ears and their infections with the pre-induced *Agrobacterium* cells was done as described by Negrotto *et al.*, (2000), except that some immature embryos was subjected physical micro-wound treatments or vacuum infiltrations before and during the infection . For each physical micro-wound treatment, 30 wounded-embryos was sub-merged into the bacterial suspension for 5-10 min; as a control, 30mm damaged embryo was infected as well. Additionally, in order to allow

the bacterial cells to penetrate into the sub peripheral host cells, 28mm Hg of vacuum infiltration was applied, during the infection step, to 30 wounded embryos generated by each physical micro-wound treatment, and to 30 non-damaged embryos used as a control. After infection, embryos was transferred to the surface of co-cultivation medium and incubated here for 3-5 days at 20^0C in the dark; after which, embryos was transferred to resting medium for 7 days (27^0C dark) putatively transformed plants was regenerated from kanamycin-resistant calli by transferring tissues to magenta boxes containing regeneration medium, supplemented with BAP 0.5 mgL^{-1} and KIN 0.5mgL^{-1}, 27^0C with a 16:8 light: dark photoperiod. After one week, an embryo was transferred to the first selection (50 mgL^{-1} kanamycin) for 3-4 weeks until the first putative transformed calli began to emerge. The *Agrobacterium* hyper-virulent strain LBA4404 has shown to be highly efficient for transforming to maize line MU-2092 and other important cultures as soya bean and rice. Progeny from some independent events generated from all the transformation procedures was analyzed by Gus histochemical or molecular analysis to determine goodness-of–fit to the expected 3:1 Mendelian ratio for self-pollinations. It was demonstrated that the introduced genes was inherited as a single mendelian locus in all events.

Efficiency of *Agrobacterium* transformation on immature embryo.

The transformation efficiency of *Agrobacterium* LBA4404 on the tropical maize line was evaluated by the transient GUS expression showed by the infected embryos. Results indicate that the LBA4404 strains was able to generate GUS –positive embryos in all tested. According with mentioned results, the following experiment was performed using the strain LBA4404 at O.D 550_{nm}0.5-1.0 and transformation efficiency was shown in Table 3.

Biolistic gene gun: Particle gun wounding treatments (bombardment) was done as described by Brett Schneider *et al*., (1997). The explant was arranged with the embryo axis exposed on the surface of the infected medium (bacterial–free) amended with 15gL^{-1} Agar, around the perimeter of a 1cm circle in 60 mmx20mm petriplates. Particle–bombardments are performed using a particle inflow Gun-PIG applying 28mmHg vacuum as described by Droste *et al*., (2000). Helium pressures of 60, 80 and 120 lb/in^2 was evaluated and all the shots was done at a distance of 19cm from the stopping screen using 1.8µm average diameter tungsten particles (GE, Cleveland). Unchanged particle is incubated with the bacteria soon after particle bombardment was performed. The transformation efficiency of maize was given in Table 4.

Effect of *Agrobacterium* transformation by physical wound and particle bombardment on transient Gus expression of maize.

The effect of the applied physical treatments was evaluated taking into account the rate of GUS positive embryos and the percentage area of transient GUS expression. When vacuum infiltration was applied, improved frequencies upto 11.6 and 22.9% of GUS-positive embryos was obtained and particle wounded embryos respectively. An enhanced percentage of transient blue area of 20.2% and 23.6% was observed in particle-wounded embryos bombarded at 80 and 120 lb/in^2 respectively. Fernandez S *et al* (1999) reported similar results when infiltration was applied for 1h in Agro-infected wheat inflorescences. However, differences in the location of blue staining was found in these treatments; while in non-damaged embryos the transient GUS expression was visible at the embryo axis, in the particle-wounded embryos the blue staining was located mainly in the central region of embryo. This could

Table 3: Efficiency of *Agrobacterium* transformation on maize.

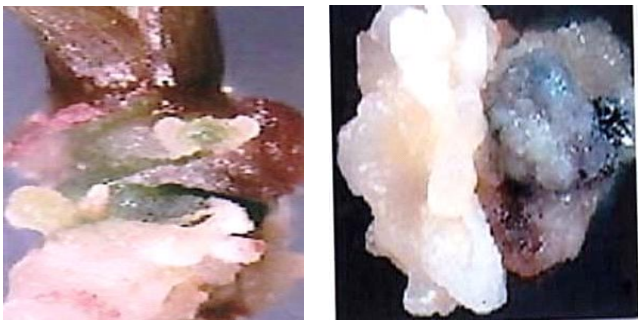
Experimental day	Embryos infected	Callus events expressing gus	Events regenerated	Transformation efficiency
Agrobacterium				
03.03.2015	21	2	2	9.5
07.04.2015	82	8	8	9.7
11.05.2015	63	4	4	6.3
22.06.2015	146	12	11	7.5
17.07.2015	126	10	8	6.3
20.08.2015	155	15	14	9.0

Table 4: Efficiency of transformation in particle bombardment of maize.

Experimental day	Embryos infected	Callus events expressing gus	Events regenerated	Transformation efficiency
Particle bombardment				
03.03.2015	21	4	4	19.0
07.04.2015	82	12	12	14.6
11.05.2015	63	7	7	11.1
22.06.2015	146	18	18	12.3
17.07.2015	126	22	22	17.4
20.08.2015	155	35	35	22.5

impact the generation of transformed embryogenic callus induced. Using maize elite inbred lines, Lupotto *et al*., (2004) reported that in all the cases in which pre-bombarded embryos are used, increases the infectivity of the *Agrobacterium* without altering the capability of the infected tissue to develop an embryogenic callus. Increases in the transformation frequencies of tobacco leaf explants and sunflower meristems by using a transformation protocol based on particle-wounded/*Agrobacterium* treated tissues was reported by Bidney *et al*., (1992). Similarly, Droste *et al*., (2000) reported upto 220 blue foci per infected clumb when using particle wounded soyabean embryogenic clumbs prior to Agro-infection, while transient GUS expression was not detected in non-damaged ones. At the present work, decreases of transient GUS expression was observed in particle-wounded embryos by bombardment at 120 lb/in^2, which could be due to the magnitude of the damage generated due to the particles penetrating the cells. Some protocols represent the addition of acetosyringone (AS) to *Agrobacterium* transformation by physical wounds lead to increase in the number and distribution of cells having transient GUS expression (Santare´m *et al*., 2003; Weber *et al*., 2003). Additionally, the wounded-induced plant cell elicitors could lead to a higher expression of the vir-genes. (Initially induced by Acetosyringone).

Histochemical Gus assays: Transient and stable gus activity studies was carried out on immature embryos 3 days after co-cultivation according with Jefferson *et al*.,(1987). An immature embryo was placed in a Gus assay mix; after 5min of vacuum infiltration, the reaction was incubated overnight at 37^{0}C. Gus activity was determined by placing the embryos on a grid and estimating the percentage of the embryos surface that showed blue sectors. Histo-chemical gus assay was also used to assess stable expression of the Gus gene both in resistant calli samples and transformed leaf tissues which was visualized by removing the Gus assay mix and rinsed twice with 70% ethanol (Figure 4).



Callus Gus Expression

Fig 4: Showing regeneration callus and callus with GUS expression.

Molecular analysis and progeny segregation: Total genomic DNA was isolated from approximately 1g of fresh leaf tissue from putative transformed and control non-transformed plants as described by Bohorova *et al.,* (1995). Molecular analysis of the 20 surviving T_0 transformants (maintained in the glasshouse) by PCR for confirmation of integration of *hpt* gene revealed that only 14 plants (P-1, P-4, P-5, P-6, P- 7, P-9, P-10, P-11, P-12, P-13, P-14, P-17, P-18 and P-20) were positive for amplification of the *hpt* gene. To establish segregation ratios for expression of the Gus gene in progeny by Gus assay test was applied. Transgenic (T_0) plants were self -fertilized. The transgene integration pattern in the nuclear genome of the putative transformed T_0 plants was to be confirmed further through Southern hybridization

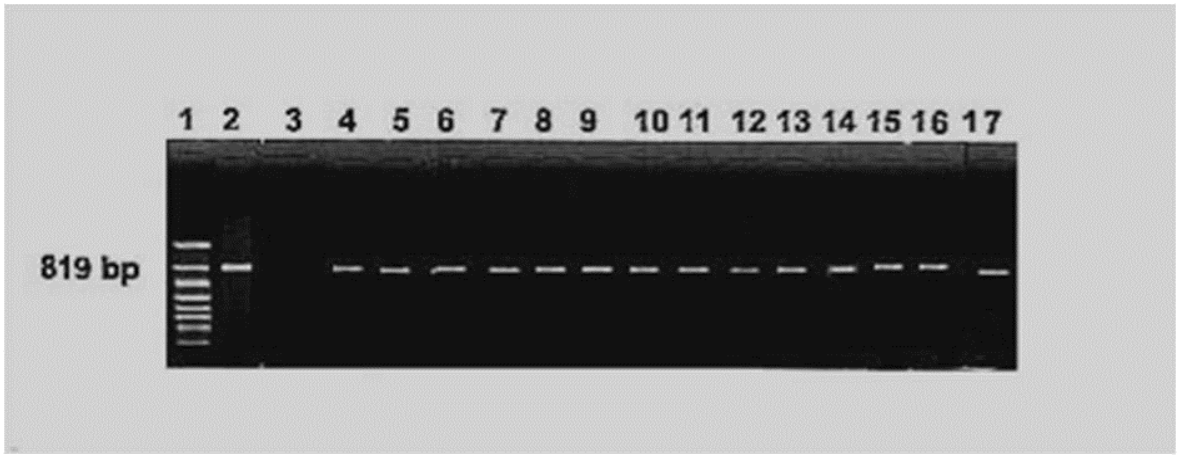


Fig 5: PCR amplification of the genomic DNA of maize MU-2092 T_0 transformants showing amplification of the 819 bp fragment of *hpt* gene.

Lane 1: DNA size marker (50 bp Marker).
Lane 2: Plasmid pCAMBIA-1301 (positive control).
Lane 3: Untransformed plant (negative control).
Lane 4 to 17: T_0 transformants (P-1, P-4, P-5, P-6, P- 7, P-9, P-10, P-11, P-12, P-13, P-14, P-17, P-18 and P-20)

of the genomic DNA (Figure – 5). Molecular characterization of later generations would be necessary for further confirmation of the transgenic nature and inheritance pattern of the transgene in the maize transformants produced in this study.

CONCLUSION

A successfully transformation procedure was developed, in which transgenic tropical maize plants could be directly generated without preliminary crosses with laboratory and/or temperate lines. This is immature embryo (target tissue) and optimization of the *Agrobacterium tumefaciens* mediated gene delivery towards immature embryo by applying physical wound prior to the agro-infection. Here, the genotype–dependence is still an important issue involved in the *Agrobacterium* maize transformation. The second is particle bombardment is used in this experiment and results are evaluated. Some other strategies reported here could be useful and get us an additional parameter to evaluate. To our knowledge, this is the best report in which the *Agrobacterium* mediated transformation technology is being extended towards the tropically adapted maize; it opens the way to generate transgenic plants in various agronomical, nutritional and nutraceutical traits in a simple and short time way.

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