CALLUS INDUCTION AND PLANT REGENERATION FROM IMMATURE EMBRYOS CULTURE OF TROPICAL MAIZE

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ABSTRACT - The development of protocols to overcome the current limitations of callus induction and in vitro regeneration of highly recalcitrant tropical maize is crucial for plant genetic transformation. The ability of embryogenic callus (EC) formation of 46 tropical maize hybrids and 14 inbred lines was tested using N_6 medium with the following changes: medium A (N_6 + 15 µM dicamba + 25 mM L-proline + 88 mM $AgNO_3$), medium B (N_6 + 30 µM dicamba + 25 mM L-proline + 88 mM $AgNO_3$), medium C (N_6+ 30 µM dicamba + 6 mM L-proline), and medium D (N_6+ 30 µM dicamba + 25 mM L-proline). Compact (Type I) and friable (Type II) callus were induced in the four media. Twenty genotypes produced callus in all media, and four genotypes (CO32, AG8012, CMS477BC_4F_2, and CMS-HGZ10) produced the highest number of callus (114, 134, 131, and 126 calli, respectively). All immature embryos of ten genotypes produced callus in at least one medium, while eight genotypes were highly recalcitrant and they did not produce any callus. The frequencies of EC ranged from 0% to 38%, and the highest rate of EC was observed on medium B (0.40) with a total of 865 calli, and the lowest induction rate was obtained with medium D (0.29) with 555 calli (P= 0.05). From the seventy-two EC of 26 genotypes transferred to Murashige & Skoog regeneration medium, twenty-four (66.7%) differentiated into green plants which produced seeds in R0 and R1 generations, and twelve (33.3%) developed into albino plants. The results demonstrated that the problem of the recalcitrant genotypes can be, at least partially, overcome by using immature embryos as explants together with tissue culture media formulations.
Keywords: *Zea mays*, immature embryos, somatic embryogenesis, tissue culture, type I and type II calli.

INDUÇÃO DE CALO E REGENERAÇÃO DE PLANTAS DA CULTURA DE EMBRIÕES IMATUROS DE MILHO TROPICAL

RESUMO - O desenvolvimento de protocolos para superar as limitações atuais de indução de calos e regeneração in vitro de milho tropical altamente recalcitrante é crucial para a transformação genética de plantas. Foi testada a capacidade de formação de calos embriogênicos (CE) de 46 híbridos de milho tropicais e 14 linhagens cultivadas em Meio N6 com as seguintes alterações: Meio A (N6 + 15 µM dicamba + 25 mM L-prolina + 88 mM AgNO3), Meio B (N6 + 30 µM de dicamba + 25 mM de L-prolina + 88 µM de AgNO3), Meio C (N6 + 30 µM de dicamba + 6 mM de L-prolina) e Meio D (N6 + 30 µM de dicamba + 25 mM de L-prolina). Calos compactos (tipo I) e friáveis (tipo II) foram induzidos nos quatro meios de cultura. Vinte genótipos produziram calos em todos os meios, e quatro genótipos (CO32, AG8012, CMS477BC4F2 e CMS-HGZ10) produziram o maior número de calos (114, 134, 131 e 126 calos, respectivamente). Todos os embriões imaturos de dez genótipos produziram calos em pelo menos um meio, enquanto oito genótipos foram altamente recalcitrantes e não produziram nenhum calo. As frequências de CE variaram de 0% a 38%, e a maior taxa de CE foi observada no meio B (0,40), com um total de 865 calos, e a menor taxa de indução com o meio D (0,29), com 555 calos (P = 0,05). Dos setenta e dois CE de 26 genótipos transferidos para meio de regeneração Murashige & Skoog, vinte e quatro (66,7%) se diferenciaram em plantas verdes que produziram sementes nas gerações R0 e R1, e doze (33,3%) se desenvolveram em plantas albinas. O problema relacionado dos genótipos recalcitrantes de milho pode ser, pelo menos parcialmente, contornado com o uso de explantes de embriões imaturos juntamente com a formulação dos meios de cultura.
Palavras-chave: *Zea mays*, embriões imaturos, embriogênese somática, cultura de tecidos, calos tipo I e tipo II.

Maize is the second largest crop in Brazil surpassed only by soybean. The crop accounts for about 30% of the Brazilian agricultural area, with production estimates of 80 million tons of grains per year in two growing seasons (Oliveira Neto, 2018). However, nutrient-poor and acidic soils, insect pests, and diseases represent a serious threat to the Brazilian agriculture, which impose the adoption of new approaches to face this challenge.

The genetic transformation is a reliable strategy for introducing foreign genes into plant chromosomes *in vitro* (Quispe-Huamanquispe et al., 2017). However, maize plants are difficult to genetically engineer, mainly due their inherent recalcitrance toward the in *vitro* culture conditions (Altpeter et al., 2016**).** The identification of maize genotypes with high ability of callus production and high green-plant regeneration ability is a necessary initial step for the subsequent genetic transformation by any of the currently available methods, namely particle bombardment, protoplast transformation, *Agrobacterium* mediated-infection and *in planta* transformation (Buggs et al., 2017; Yadava et al., 2016).

The majority of successful protocols for plant transformation using callus induction and plant regeneration in maize have been developed for non-commercial inbred lines with low agronomic performance and adapted to temperate climates (Lu et al., 1982). Thus, crosses through conventional plant breeding methods are required to transfer the foreign genes to elite lines. The use of highly regenerative tropical genotypes selected from high-value commercial varieties can reduce the costly and time-consuming breeding programs to transfer the genes from one variety to another.

Many authors started to evaluate the potential of callus formation and plant regeneration of tropical-adapted maize thorough indirect somatic embryogenesis using immature embryos (Bohorova et al., 1995; Carvalho et al., 1997; Petrillo et al., 2008). In tropical maize, the ability of a given genotype to induce Type I (compact) or Type II (friable) callus and regenerate plants are genetically determined with frequencies ranging from 4% to 32.5% (Al-Abed et al., 2006). However, Akoyi et al. (2013) reported that embryogenic and non embryogenic callus induction was independent of the genotype when young immature embryos were cultured in medium supplemented with Dicamba. In addition, results of other studies have also shown that callogenesis and somatic embryogenesis in maize are also determined by biochemical parameters of the medium, such as the carbon and nitrogen sources, the type and concentration of growth regulators (auxins, gibberellins, cytokinins, abscisic acid and ethylene), L-proline, silver nitrate, and even the orientation of the embryos in the culture medium (Azad et al., 2015; Lu et al., 1982; Muli et al., 2017). Among the growth regulators, the auxin-like 2,4-dichlorophenoxyacetic (2,4-D) was considered the most efficient for embryogenic callus induction and plant regeneration in cereal crops. This is probably because it is metabolized more slowly compared to other auxins, and its role in activating the transcription of the somatic embryogenesis receptor kinase gene responsible for somatic embryogenesis in many plant species (Ombori et al., 2008). Differently, some authors reported that immature embryos showed better callus response and produced more somatic embryos in medium supplemented with the auxin-like dicamba herbicide than in the medium containing 2,4-D (Furini & Jewell, 1994; Rakshit et al., 2010).

In the present work were evaluated the effects of four culture medium formulations on callus

induction and plant regeneration ability of sixty maize genotypes adapted to the Brazilian tropical climate.

Material and Methods

Plant materials

In this study, a total number of 60 tropical-adapted maize genotypes were evaluated (Table 1). Forty-four sib-pollinated hybrids of tropical maize from seeds companies and assigned into three groups according to cycle length: 12 early-maturity, 22 intermediate and 10 late-maturity hybrids; eleven inbred lines selected for high levels of gamma-zein accumulation in the endosperm, and five inbred lines from the Embrapa Maize and Sorghum breeding programs.

Explant preparation and culture conditions

Sixteen to twenty days after pollination, ears were harvested and surface-sterilized by immersion in

Table 1 – List of maize genotypes and their genetic origins.

Genotype	Origin	Genotype	Origin
CATETE	Embrapa Maize & Sorghum	AG 8012	Commercial hybrid
BR 111 - Pool 21 CIMMYT	Embrapa Maize & Sorghum	AG 5011	Commercial hybrid
CMS 14 - Pool 25 CIMMYT (298)	Embrapa Maize & Sorghum	AG 1051	Commercial hybrid
CMS 14 - Pool 25 CIMMYT (299)	Embrapa Maize & Sorghum	AG 1043	Commercial hybrid
CMS 52 - Experimental Variety	Embrapa Maize & Sorghum	AG 951	Commercial hybrid
HT 16C	Embrapa Maize & Sorghum	DINA 887	Commercial hybrid
BR 106 (12)	Embrapa Maize & Sorghum	DINA 888	Commercial hybrid
CMS 477 BC_4 F_2	Embrapa Maize & Sorghum	DINA 657	Commercial hybrid
CMSHGZ04	Embrapa Maize & Sorghum	X-9456	Commercial hybrid
CMSHGZ05	Embrapa Maize & Sorghum	AS 22	Commercial hybrid
CMSHGZ08	Embrapa Maize & Sorghum	RA 100	Commercial hybrid
CMSHGZ09	Embrapa Maize & Sorghum	Z 8202	Commercial hybrid
CMSHGZ10	Embrapa Maize & Sorghum	Z 8452	Commercial hybrid
CMSHGZ15	Embrapa Maize & Sorghum	Z 8501	Commercial hybrid
CMSHGZ17	Embrapa Maize & Sorghum	AGN 3150	Commercial hybrid
CMSHGZ18	Embrapa Maize & Sorghum	AGN 2014	Commercial hybrid
C 806	Commercial hybrid	AGN 1040	Commercial hybrid
C 909	Commercial hybrid	OC 358101	Commercial hybrid
C 615	Commercial hybrid	OC 705	Commercial hybrid
C 444	Commercial hybrid	EXCELER	Commercial hybrid
C 333 B	Commercial hybrid	G 165 S	Commercial hybrid
CO-9509	Commercial hybrid	A 952	Commercial hybrid
CO 32	Commercial hybrid	X HT 12	Commercial hybrid
P 3072	Commercial hybrid	FT 9006	Commercial hybrid
P 3081	Commercial hybrid	PL 303	Commercial hybrid
P 3041	Commercial hybrid	M 9560	Commercial hybrid
P 3027	Commercial hybrid	IAC EXP. 4243	Commercial hybrid
P 3232	Commercial hybrid	AL 25/11	Commercial hybrid
AG 6014	Commercial hybrid	ALP 92-59	Commercial hybrid
AG 3010	Commercial hybrid	AL Manduri	Commercial hybrid

70% ethanol, followed by 2.5% sodium hypochlorite for 40 min and rinsed three times with sterile deionized water. Immature embryos 1.0-1.5 mm long were excised from the kernels under aseptic conditions with a scalpel and spatula. Twelve to fourteen embryos per plate were placed, flat surface down, with the scutellum side up on four distinct callus initiation media, and the cultures were incubated at 27°C, in the dark for two to three weeks.

Culture media

The basic medium CM-1 for embryo culture consisted of N_6 inorganic salts (Chu et al., 1975), sucrose (30,000 mg L^{-1}), casamino acids (100 mg L^{-1}), myo-inositol (550 µM), glycine (30 mM), thiamine-HCl (15 µM), pyridoxine-HCl (7.5 µM), and nicotinic acid (7.5 µM). Four variants of the CM-1 medium differing by dicamba, L-proline and silver nitrate concentrations are shown in Table 2. All media were solidified with phytagel (1.8 g L^{-1}), and the pH was adjusted to 5.8 with KOH before autoclaving.

The average percentage of callus formation was scored for all genotypes and each medium tested. Embryogenic calli were maintained by subculturing at every 21 days on the same callus initiation medium. Both embryogenic calluses, Type I (hard, compact, nodulated, and yellowish tissue) and Type II (soft, friable, and white-to-brown) were used for plant regeneration.

Plant regeneration

Approximately four to five months after callus formation, small pieces (~5mm) of embryogenic callus were transferred to the regeneration medium consisting of MS (Murashige & Skoog, 1962) basal medium without any growth regulators, and supplemented with myo-inositol (550 µM), glycine (30 mM), thiamine-HCl (15 µM), pyridoxine-HCl (7.5 µM), nicotinic acid (7.5 µM), sucrose (20,000 mg L^{-1}), and 1.8 g L^{-1} phytagel, pH 5.8. After four weeks of incubation, the plantlets were transferred to the rooting medium, which consisted of the regeneration medium supplemented with 1.0 mg/L a-naphthalene acetic acid (NAA) and 8g/L agar. After rooting, the plantlets were transferred to plastic pots containing soil/vermiculite (2:1) mixture and kept in the greenhouse under high humidity level.

Statistical analysis

Statistical analysis to determine frequencies (%) of callus induction, somatic embryogenesis, and plant regeneration was performed using SAS (Statistical Analysis System) version 9.2 (2008). Tukey's HSD (Honestly Significant Difference) test at 95% confidence interval was used to determine significant differences among the parameters under study.

Table 2 - Media composition for callus induction from maize immature embryos.

Type of medium	Medium Description
A	CM-1* + 15 µM dicamba + 25 mM L-proline + 88 µM $AgNO_3$
B	CM-1 + 30 µM dicamba + 25 mM L-proline + 88 µM $AgNO_3$
C	CM-1 + 30 µM dicamba + 6 mM L-proline
D	CM-1 + 30 µM dicamba + 25 mM L-proline

*CM-1 = N6 Basic medium (Chu et al., 1975).

Results and Discussion

Callus formation directly from the scutellum of maize immature embryos was observed by approximately forty days after culture initiation (Figure 1A). Callus production was observed on the four media tested (Table 2), and was highly dependent on the genotype (Figure 1B, C and D). The capacity of maize to produce regenerable callus from immature embryos is determined by quantitative trait loci (QTL) with epistatic interactions, and additive genes with positive heterotic effects (Tomes & Smith, 1985). Two types of embryogenic calli (type I and type II) were reported in maize tissue culture by Tomes and Smith (1985). In the present study, the number of regenerated plants from both types of embryogenic callus was very similar, thus they were considered together. The total percentage of callus formed ranged from 29% on media D to 40% on medium B (Table 3). The type I callus was compact, nodular, and white to cream colored, with slow growth and proliferating as a mixture of complex tissues showing somatic embryos. Callus Type II was light yellow, highly friable, soft and characterized by fast growth. The type II callus has a high embryogenic activity rate, which permits its cultivation for a long period (over a year) as reported by Tomes and Smith (1985). According to Al-Abed et al. (2006) and Ombori et al. (2008), this characteristic of callus type II was due to the existence of meristematic cells in the scutellum of maize embryos.

The higher average percentage (40%) of callus formation for all genotypes tested was observed in the medium B (Table 3). The medium D, which was similar to medium A but lacking $AgNO_3$, was the less efficient for callus induction (29%). This data stresses the favorable effects of silver cation (Ag^+) on callus induction and plant regeneration potential in tropical genotypes of maize (Bohorova et al., 1995; Rakshit et al., 2010). However, the cellular role of Ag^+ on cultured tissues remains unknown. Beyer (1976) speculated that silver ions may act as inhibitors of the ethylene action, which in turn inhibits auxin transport in plants.

In the present study, the influence of the auxin-like dicamba on callus induction could be observed comparing the data of Medium A (15 µM dicamba) and Medium B (30 µM dicamba) (Table 3). The average of embryos producing callus in the Medium B and Medium A were about 40% and 37%, respectively. Although the difference between these two treatments was not statistically significant, calli formed on medium B were more friable and embryogenic than those of Medium A. This result may be attributed to differences in the concentration of dicamba in the media. In fact, dicamba has been postulated as the best regulator for callus initiation and maintenance in tropical maize (Bohorova et al., 1995). Contrary, Anami et al. (2010) found that the 2,4-dichlorophenoxyacetic acid (2,4-D) concentration at the callus initiation stage from immature embryos is determinant for shoots regeneration in tropical maize.

In relation to the addition of the amino acid L-proline in the medium, studies reported that the presence of this amino acid in the culture promoted embryogenesis and plant regeneration in somatic tissues of maize (Armstrong & Green, 1985) and other species (Ozawa & Komamine, 1989). In our study, however, we could not observe any improvement on callus formation due the addition of L-proline to the media. In fact, different concentrations of L-proline in medium C (6 mM) and medium D (25 mM) showed similar results with averages of 32% and 29% of callus formation, respectively.

Table 3 - Average percentage (%) of callus formation from maize immature embryos in four different culture media.

Genotypes	Medium			
	A	**B**	**C**	**D**
CATETE	42e	52d	38e	46f
BR 111 Pool 21 CIMMYT	35e	12h	24f	41f
CMS 14 Pool 25 CIMMYT (298)	37e	86c	72c	00i
CMS 14 Pool 25 CIMMYT (299)	34e	56d	34f	00i
CMS 52- Experimental Variety	22f	43e	77c	00i
HT 16 C	48d	92b	79c	50e
BR 106(12)	06g	08h	00h	00i
CMS 477 BC_4 F_2	65c	50d	100a	100a
CMS-HGZ04	00g	00i	00h	00i
CMS-HGZ05	100a	67c	17g	08i
CMS-HGZ08	00g	00i	00h	00i
CMS-HGZ09	00g	00i	00h	00i
CMS-HGZ10	100a	100a	00h	100a
CMS-HGZ15	00g	00i	00h	00i
CMS-HGZ17	00g	46e	78c	00i
CMS-HGZ18	00g	00i	00h	00i
C806	70c	70c	00h	00i
C909	58d	17h	39e	00i
AG 3010	00g	36g	96b	04i
Z 8202	25f	00i	26e	13h
AGN 3150	42e	100a	58f	00i
CO 32	25f	76c	79c	100a
P3072	02g	00i	00h	00i
P3081	63d	38f	58d	83b
AG 8012	92b	86c	88c	100a
OC 358101	38e	25g	75c	50e
Exceler	33e	67c	00h	00i
A 952	00g	00i	05h	00i
Dina 888	00g	60d	67e	50e
XHT 12	00g	05i	00h	00i
P 3041	63d	82c	77c	00i
AGN 2014	71c	42f	00h	00i
Z 8452	50d	100a	00h	00i
FT 9006	42e	58d	67c	55e
P 3027	71c	57d	68c	33f
PL 303	00g	00i	00h	27g
G 165 S	08g	04i	00h	00i
C 615	09g	08h	04h	16h
M 9560	08g	00i	00h	00i
IAC EXP. 4243	67c	88b	17g	42f
AL 2511	00g	100a	00h	00i
C 444	09g	09h	17g	100a
AG 5011	25f	04i	08h	00i
Dina 657	00g	14h	09h	00i
X9456	88b	79c	38e	55h
AS22	50d	33f	29f	37g
RA 100	00g	17h	09h	00i
OC 705	00g	00i	00h	00i
AGN 1040	00g	00i	05h	38f
Z 8501	17f	09h	00h	00i
P 3232	57d	05i	00h	00i
AG 6014	63d	42f	63d	54e
ALP 9259	05g	16h	00h	00i
AG 1051	36e	04i	17g	55e
AG 1043	00g	13h	00h	00i
AL Manduri	21f	04i	00h	39f
AG 951	52d	30g	25f	75c
DINA887	54d	75c	59d	71d
C 333B	00g	33g	00h	00i
CO-9509	38d	38d	42a	00i
Average	**37**	**40**	**32**	**29**

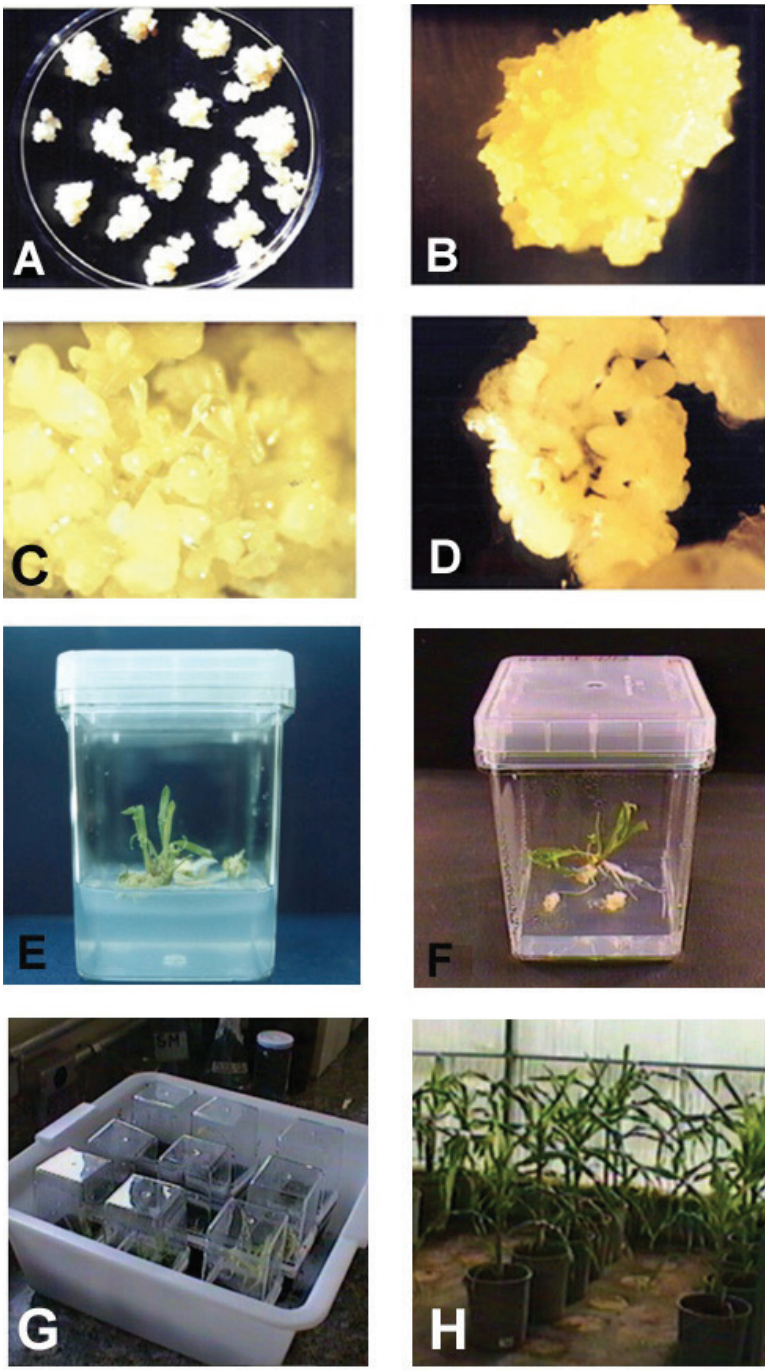

Figure 1. Somatic embryogenesis and plant regeneration from immature embryos of tropical maize hybrid C909. A and B callus initiation on medium B. Embryos explants showing callus initiation after 14 days in culture (A). Embryogenic callus (Friable calli Type II) at the globular stage (B). Differentiation of heart-shaped somatic embryos in N6 medium supplemented with 6% sucrose and 1 mg L^{-1} NAA (C and D). Shoot development from the embryogenic callus on MS medium without growth regulators (E). Root development in regenerated plantlets (F). Embryogenic R0 plants acclimatization in the greenhouse 4 months after culture initiation (G), and mature R1 plants (H).

In this study, the best medium/genotype combination, regard callus production, was observed for the medium B and the two commercial hybrids, AG 8012 and IAC Exp 4243. Among all hybrids tested we could not notice any influence due to the plant cycle length (early-maturity, intermediate and late-maturity) on callus formation ability *in vitro*. Among genotypes with high content of gamma-zein, only three (CMS477, CMSHGZ08 and CMSHGZ18) showed embryogenic type II callus formation. However, none of these three genotypes regenerate plants. Among all 60 genotypes evaluated, only 26 formed embryogenic calli.

In our study, dark embryos without any callus were discarded after 45 days of cultivation and subculturing at three week intervals. Seventy-two embryogenic calli with somatic embryos transferred to regeneration medium turned green and developed vigorous shoots. The commercial hybrids C909 and Dina 887 also produced a good root system after 30 days on regeneration medium (Figure 1E and 1F), and some somatic embryos did not grow further to form plantlets. As previously postulated by Ikeda-Iwai et al. (2003) and Ombori et al. (2008), the down-regulation of genes responsible for plant regeneration from immature embryos-derived callus could explain the differences in tissue culture responses among genotypes. This may also explain the absence of plant differentiation of some somatic calli in our study. In addition, shoot formation from embryos-derived callus was significantly higher in hybrids C909 and Dina 887 compared to those formed by the other genotypes. This result showed that plant regeneration was genotype-dependent, as previously demonstrated in many cereal species (Anami et al., 2010; Bohorova et al., 1995; Ombori et al., 2008; Tomes & Smith, 1985).

In vitro regenerated plants (R0) with well-developed roots were transferred to pots with soil and vermiculite mixture for further growth in the greenhouse (Figure 1G and Table 4). Seeds of R0 plants grown to maturity in the green house and R1 seeds generated new plants (Figure 1H). Some genotypes generated only plants with small shoots, and they were not able to develop roots, even after 30 days on rooting medium (1.0 mg L^{-1} NAA).

At the end of the process, nineteen plants were transferred to greenhouse and all of them produced seeds normally when transferred to green house. The efficiency of the process was of 26.39% (19 plants from 72 embryoids).

Table 4 - Morphogenic responses of maize callus in the regeneration medium.

Morphogenesis							
Embryogenic callus	Plant regeneration	Stem		Plant		Seedling rooting	Process efficiency
		(+)	(-)	Albinos	Green		
72	36 (50%)	19/36 (52.8%)	17/36 (47.2%)	12/36 (33.3%)	24/36 (66.7%)	19/36 (76.0%)	19/72 (26.39%)

(+) = presence; (-) = absence

Conclusions

The present results indicated that somatic embryogenesis of recalcitrant maize genotypes and plant regeneration can be, at least partially, overcome by using immature embryos as explants and culture media formulation.

In this study, we have implemented and optimized a reliable protocol for callus induction and plant regeneration through somatic embryogenesis of immature maize embryos and culture media formulation. The efficiency of the process was of 26.39%.

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