Somatic embryogenesis and plant regeneration capacity in Argentinean maize (*Zea mays* L.) inbred lines

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Abstract Somatic embryogenesis, which is still the method of choice for tissue culture, regeneration and transformation of maize, is largely considered highly genotype-dependent. The Hi II, a highly embryogenic genotype, has been extensively used in transformation protocols. However, this is not an inbred line; instead, it has a proportion of the undesirable A-188 background, and the progeny segregates for phenotypic characteristics and shows poor agronomic performance. In an effort to identify genotypes that combine a high somatic embryogenic response and good agronomic performance, we evaluated 48 advanced inbred lines developed at INTA. Callus development and somatic embryogenesis capacity were measured in 200 immature embryos per line. Embryogenic capacity [EC (mature somatic embryos/callus evaluated) x 100], Regeneration Capacity (RC) and Fertile Plant Recovery in greenhouse (FPR, fertile plants/regenerated plants) were recorded. A total of 17 lines reached an EC > 50%, and 14 out of those 17 lines regenerated seedlings. The FPR ranged between 50 and 100%. Also, we selected three promising lines with high agronomic performance, as alternatives to Hi II, in order to be included in a maize transformation scheme *via* somatic embryogenesis. In addition, we report the usefulness of Single Sequences Repeat (SSRs) in the determination of genetic diversity among 14 divergent lines for somatic embryogenesis response. The seven lines displaying good *in vitro* behaviour can be crossed to obtain hybrids combining desirable alleles for somatic embryogenesis response and different genetic backgrounds.

Keywords: callus, inbred lines, maize, molecular markers, regeneration, somatic embryogenesis

INTRODUCTION

The system based on somatic embryogenesis has been the method of choice for tissue culture, regeneration and transformation of maize for over twenty years. Somatic embryogenesis plays an important role in clone propagation. When integrated with conventional breeding programs and molecular and cell biology techniques, somatic embryogenesis provides a valuable tool to enhance the progress of commercial crop species.

Somatic embryogenesis involves the establishment of an explant in culture, the subsequent proliferation of calluses, and initiation of somatic embryos (von Arnold et al. 2002). Green and Philips (1975) first described plant regeneration in maize and obtained embryogenic calluses derived from immature embryos of the A188 inbred line. Additionally, two types of embryogenic calluses have been described for maize: Type I, which are compact and more easily obtained from immature embryos, and

Type II, which are friable and maintain the capacity to regenerate plants over a longer period of time (Wang et al. 2009).

Bohorova et al. (1995) opened the discussion about the capacity to produce regenerable callus lines from immature embryos that appear to be genotype-dependent in many species, including maize (Che et al. 2006; Wang et al. 2009). Some reports have indicated that regeneration in maize is genetically controlled by nuclear genes (El-Itriby et al. 2003). In addition, Quantitative Trait Loci (QTL) associated with the induction of embryogenic calluses, including QTL with main effects and epistatic interactions (Krakowsky et al. 2006), have been identified in maize.

Regeneration efficiencies from calluses are low for most agronomically useful inbred lines publicly available (*e.g.,* B73 and Mo17). Therefore, maize elite lines remain inaccessible to improvement by applying the direct transformation technique, because they fail to produce embryogenic calluses from competent tissues or to regenerate fertile plants efficiently after embryogenic callus induction. These disadvantages create the need to use less desirable inbred lines (*e.g.*, A188 and H99) for genetic transformation and plant regeneration (Krakowsky et al. 2006). At present, Hi II is the genotype used in most maize transformation protocols (Frame et al. 2000; Shou et al. 2004; Ghosh Biswas et al. 2006; Aluru et al. 2008). This line was developed to obtain high levels of Type II embryogenic calluses and calluses derived from the Hi II genotype with high regeneration capacity (Armstrong et al. 1991). However, the *ex vitro* culture of Hi II plants is very difficult because the plants show low agronomic aptitude, small ears and the sequential maturation first of pollen and then of ovules (protandry). The study of somatic embryogenesis of new genotypes will provide lines that combine high *in vitro* capacity with good agronomic performance, thus facilitating the subsequent steps of maize breeding programs.

Several efforts have been made to identify new genotypes and improved conditions to achieve high regeneration capacity (Frame et al. 2000; Danson et al. 2006; Hernández-García, 2007; Binott et al. 2008; Anami et al. 2010).

The objective of this work was to evaluate the production of embryogenic calluses and plant regeneration *via* somatic embryogenesis of several inbred lines developed in the maize breeding programs of the National Institute of Agricultural Technology (INTA), Castelar, Argentina, with the aim to identify lines for successful genetic transformation.

MATERIALS AND METHODS

Plant material

A total of 48 maize inbred lines from the breeding programs of INTA were studied in the 2008-2009 maize growing season (Table 1). These lines represent all different groups of a broader collection and their characteristics are detailed in Table 1. Plants were self-pollinated and whole ears were collected 12-14 days after pollination to assure appropriate callus induction. It is known that this developed stage of zygotic embryos corresponds to a high callus induction rate (Zhang et al. 2011). Kernels were aseptically excised and washed with Tween-20 followed by surface-sterilization with sodium hypochlorite (0.6%) for 20 min. Subsequently, immature kernels were washed five times with sterilized distilled water.

A total of 200 immature embryos *per* inbred line of 1.0-2.0 mm were aseptically excised from surface-sterilized kernels under binocular microscope into laminar flow and placed with plumule-radicle axis in contact with callus induction medium (Anami et al. 2010).

The assay was replicated the following growing season but using only the lines that had displayed the best performance at *in vitro* culture during the first season (Table 1).

Culture initiation and maintenance

Callus induction medium was based on N6 medium and vitamins supplemented with 2 mg l^{-1} 2,4-dichlorophenoxyacetic (2,4-D), 2.9 g l^{-1} L-proline, 0.1 g l^{-1} casein hydrolysate, 30 g l^{-1} sucrose, 2.5 g l^{-1}

Phytagel, 1.7 mg l^{-1} silver nitrate, autoclaved and adjusted to pH 5.8 (Wang et al. 2003). Constant conditions of this medium were used to determine which genotype had better *in vitro* behaviour (Danson et al. 2006), and could adapt *prima facie* to the *in vitro* culture and transformation protocols widely used in maize genetic transformation (Ghosh Biswas et al. 2006; Aluru et al. 2008; Décima Oneto et al. 2010; Décima Oneto et al. 2011).

Table 1. List of the 48 maize inbred lines from the breeding programs of INTA. The table presents the genotype name and the main features of each line. Kernel texture (Tex.) and Colour (Col.): SF (Semi-Flint), F (Flint), SD (Semi-Dent), O (Orange), Y (Yellow), R (Red), OY (Orange-Yellow) and W (White). Rust, Blight and Stem breakage/Root tip (Sb/Rt): very resistant (vr), resistant (r), tolerant (t), susceptible (s) and very susceptible (vs). Pollination Fitness (Poll. Fit.) and Grain yield: very good (vg), good (g), regular (r) and bad (b). Ears for Plant (Ears/Plant).

Number of line	Genotype	Tex.	Col.	Rust	Blight	Sb/Rt	Poll. Fit.	Grain yield	Ears/ Plant
1#	LP236	SF	O	vr	w/d	r	g	vg	1
2	LP214	F	O	w/d	w/d	w/d	w/d	w/d	w/d
3	LP2542	SF	Y	w/d	w/d	w/d	w/d	w/d	w/d
4	LP179	F	O	r-t	r	r	g	g	2
5	LP1513	SF	Y	w/d	w/d	w/d	w/d	w/d	w/d
6	LP122-2	F	R	r-t	w/d	r	g	r	1
7#	LP918	F	O	w/d	w/d	w/d	w/d	w/d	w/d
8	LP612	F	O	vr	r	t	g	r	1
9	LP299-2	SD	OY	t	t	t	g	vg	1 to 2
10	LP2	SF	Y	s	s	s	g	g	1
11#	LP917	SF	O	w/d	w/d	w/d	w/d	w/d	w/d
12	LP221	SF	O	w/d	w/d	w/d	w/d	w/d	w/d
13	LP613	F	O	vr	r	t	g	r	1
14	LP605	SF	O	w/d	w/d	w/d	w/d	w/d	w/d
15	LP562	SF	O	w/d	w/d	w/d	w/d	w/d	w/d
16	LP563	SF	O	w/d	w/d	w/d	w/d	w/d	w/d
17	LP463	SF	O	w/d	w/d	w/d	w/d	w/d	w/d
18*#	LP125-r	F	O	t	t	r	g	Vg	1
19*#	LP317	F	Y	t	t	t	vg	vg	1
20	LP438	SF	O	w/d	w/d	w/d	w/d	w/d	w/d
21#	LP126	SF	O	w/d	w/d	w/d	w/d	w/d	w/d
22	LP1411	SF	R	r	r	t	g	g	1 to 2
23*#	LP4703	SF	O	t	t	r	vg	g	2
24	LP561	F	R	t	t	r	g	vg	1
25	LP1512	F	O	t	s	r	g	g	1
26*#	LP509	F	R	t	t	t	g	g	1
27	LP1032	F	R	t	t	t	g	g	1
28#	LP199	F	O	t	t	r	vg	g	1
29#	LP59	F	OY	t	t	r	g	g	1
30	LP521	F	O	t	t	r	g	vg	1
31	LP256-r	F	R	vr	r	t-s	g	g	1
32	LP662	F	O	s	s	r	vg	vg	1
33	LP3830	SF	Y	t	t	r	vg	vg	1 to 2
34	LP579	F	R	s	t	t	g	g	1
35	LP5708	F	R	t	t	s	g	g	1
36*#	LP311	F	O	t	t	t	vg	vg	1
37	LP124	F	R	r	r	t	g	g	1
38	LP915	SF	O	t	t	r	g	g	1
39	LPB1	F	W	s	t	s	b	r-g	1
40	LPB2	F	W	vr	w/d	r	g	r-g	1
41	LP869	F	OY	r	r	r	g	g	1
42*#	LP1044	F	O	t	t	t-s	g	g	1
43	LP197	SD	OY	r	r	r	g	vg	1 to 2
44	LP13	F	O	t	t	t-s	g	g	1
45	LP147	F	OY	t	t	r	r	r	1
46	BLS61	F	O	w/d	w/d	w/d	w/d	w/d	w/d
47	BLS91	F	O	w/d	w/d	w/d	w/d	w/d	w/d
48#	BLS101	F	O	w/d	w/d	w/d	w/d	w/d	w/d

* Lines with good *in vitro* behaviour (CI or EC > 0.5). # Lines with poor *in vitro* behaviour (CI or EC < 0.2). Without data (w/d). Asterisk (*) indicates the lines evaluated in both growing seasons. Numeral (#) indicates the lines included in the genetic divergence analysis.

Immature embryos were incubated in the dark for 20 days at 26ºC. Then, the frequency of callus induction was determined and the callus type was classified. Likewise, the *in vitro* capacity (IC) was calculated as the frequency of calluses with embryogenic response/total calluses developed.

Calluses obtained from the lines in the second growing season were maintained in callus induction medium for three months with subculture every two weeks. The IC was observed every two weeks for each line in order to detect changes in callus performance.

Somatic embryo development and regeneration

A total of 30 calluses of each inbred line developed in callus induction medium were taken at random and transferred to somatic embryo medium. This medium was based on MS medium (Murashige and Skoog, 1962) supplemented with 0.25 mg l^{-1} naphthalene acetic acid (NAA), 60 g l^{-1} sucrose, 2.5 g l^{-1} Phytagel autoclaved and adjusted to pH 5.8. Calluses were incubated in the dark for 30 days at 26ºC. The embryogenic capacity (EC) was determined as the frequency of calluses that showed mature somatic embryos on their surface.

A total of 30 mature somatic embryos of each line were transferred to regeneration medium to obtain regenerated seedlings. This medium was similar to the somatic embryo medium but without any growth regulators, plus 30 g l^{-1} sucrose. Mature somatic embryos were incubated under a photoperiod of 16:8 hrs at 26ºC. After 2-3 weeks, the regeneration capacity (RC) was determined as the frequency of germinated somatic embryos.

All the regenerated seedlings were transferred to pots containing a 3:1 soil:vermiculite mixture and grown in growth chamber (26ºC, 16:8 photoperiod, 90% humidity) for two weeks for acclimation. Plantlets were further transferred to 5-L pots and grown in greenhouse (Décima Oneto et al. 2011). At flowering, the percentage of fertile plants recovery (FPR) was determined for each inbred line.

Statistical analysis

Statistical analysis was carried out applying ANOVA. The parameters analyzed were the IC, EC, RC and FPR. Means were calculated using INFOSTAT software (Di Rienzo et al. 2010) and compared using Tukey' test.

Histological analysis of embryogenic callus

Embryogenic calluses were fixed in FAA (absolute ethylic alcohol, glacial acetic acid, formaldehyde, and distilled water, in a 50:5:10:35 ratio v/v), dehydrated in ethylic alcohol (50, 70, 96 and 100%) and included in Paraplast (Sigma®). Cuts of 8 to 10 µm were obtained using a rotating microtome, and placed in a flotation bath (water and grenetin) at 60ºC. The samples were fixed to slides and stained with saphranine-rapid green (Iracheta-Donjuan et al. 2003), and finally mounted on synthetic resin. The samples were observed and photographed in an Olympus E-330 optical microscope with an integrated digital camera (Olympus BX-40).

Genetic diversity analysis

A subset of 14 out of 48 lines was subjected to genetic analysis to determine the level of genetic diversity (Table 1). The selection of the 14 lines was based on their extreme response to somatic embryogenesis. The inbred lines were genotyped with 50 SSR markers [name (chromosome number)]: Bnlg1643 (1), Bnlg1179 (1), Bnlg1007 (1), Dupssr25 (2), Phi104692 (2), Bnlg1047 (2), Umc1551 (2), Bnlg1601 (3), Phi099 (3), Phi073 (3), Bnlg197 (3) Bnlg1189 (4), Phi21 (4), Bnlg1434 (4), Umc1509 (4), Umc1362 (4), Bnlg1700 (5), Umc1585 (5), Bnlg1237 (5), Dupssr10 (5), Bnlg2323 (5), Phi116 (6), Phi070 (6), Phi077 (6), Phi126 (6), Bnlg480 (6) Umc1023 (6), Phi123 (6), Dupssr15 (6), Blng1808 (7), Umc1456 (7), Phi057 (7), Dupssr9 (7) Bnlg2259 (7), Phi080 (8), Umc1304 (8), Phi080 (8), Umc1741 (8), Phi115 (8), Bnlg1056 (8), Dupssr3 (8), Bnlg1352 (8), Bnlg2037 (8), Phi027 (9), Dupssr6 (9), Dupssr19 (9), Bnlg128 (9), Umc1152 (10), Bnlg1074 (10) and Umc1556 (10). At least three Single Sequences Repeat SSRs loci from each maize chromosome were assayed and the SSR for each chromosome was selected at random using the MaizeDB database marker information

(http://www.agron.missouri.edu). Genomic DNA was isolated from young leaf tissue following the protocol described by Dellaporta et al. (1983). Each 15-µL PCR reaction consisted of 50 ng of DNA, 0.2 mM of each deoxyribonucleotide, 1.5 mM MgCl2, 1X PCR Buffer (20 Tris-HCl, pH 8.0 and 50 mM KCl), 0.25 µM each primer and 1 U Taq DNA polymerase (Invitrogen, Carlsbad, USA). PCR amplifications were performed in a thermal cycler *T-Gradient* (Biometra®, Germany) using a "touchdown" program: 2 min at 94ºC, followed by 10 cycles of 30 sec at 94ºC, 40 sec at 65ºC (reduced by 1ºC per cycle), 50 sec at 72ºC, followed by 25 cycles of 30 sec at 94ºC, 40 sec at 55ºC, 50 sec at 72ºC and a final extension of 5 min at 72ºC. The amplification products were resolved by electrophoresis on denaturing polyacrylamide gels (6% acrylamide/bisacrylamide 19:1, 8M urea in TBE, pH 8.3) and visualized by silver staining (Creste et al. 2001).

The amplified fragments were scored as present (1) or absent (0) for each SSR marker allele and data were entered in a binary data matrix as discrete variables. The binary matrix was transformed into a similarity matrix using Jaccard's coefficient (Reif et al. 2005) using NTSYS-pc 2.02 software. Genetic distances were calculated as: 1-Jaccard's coefficient.
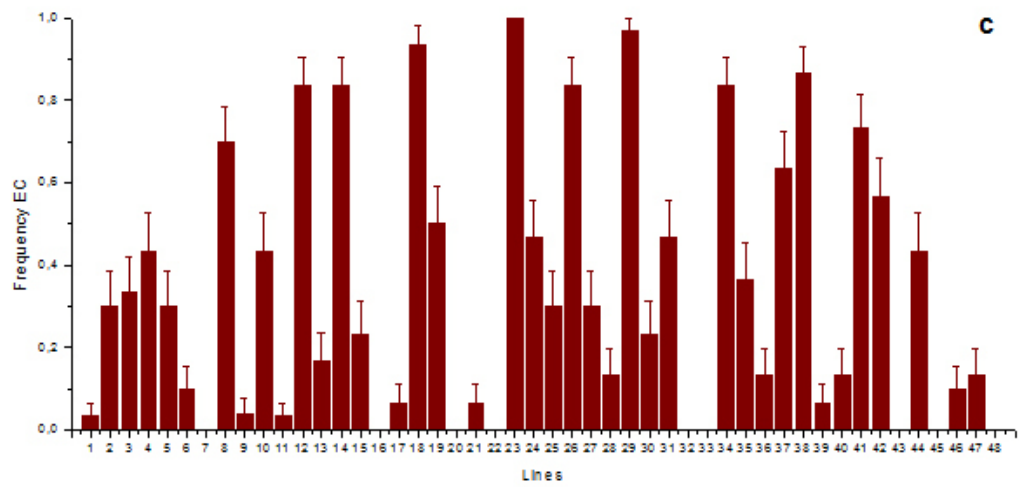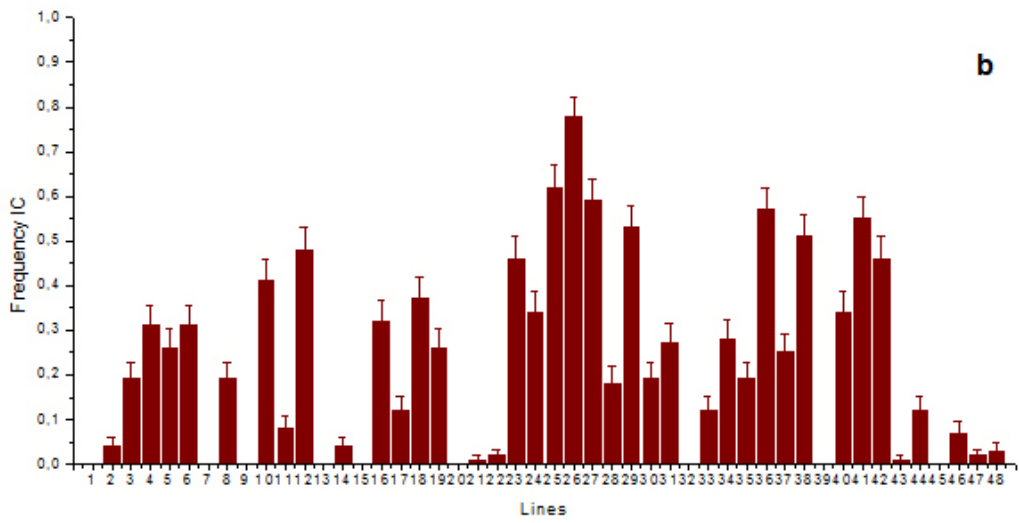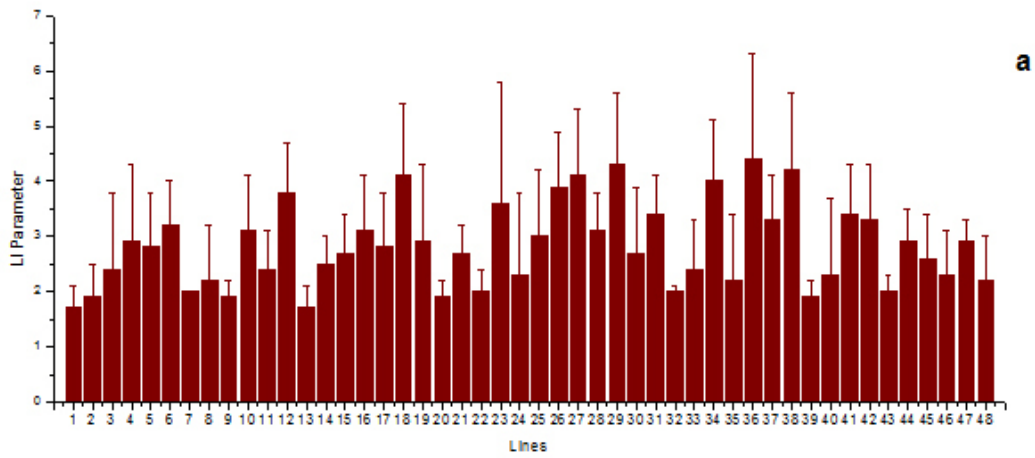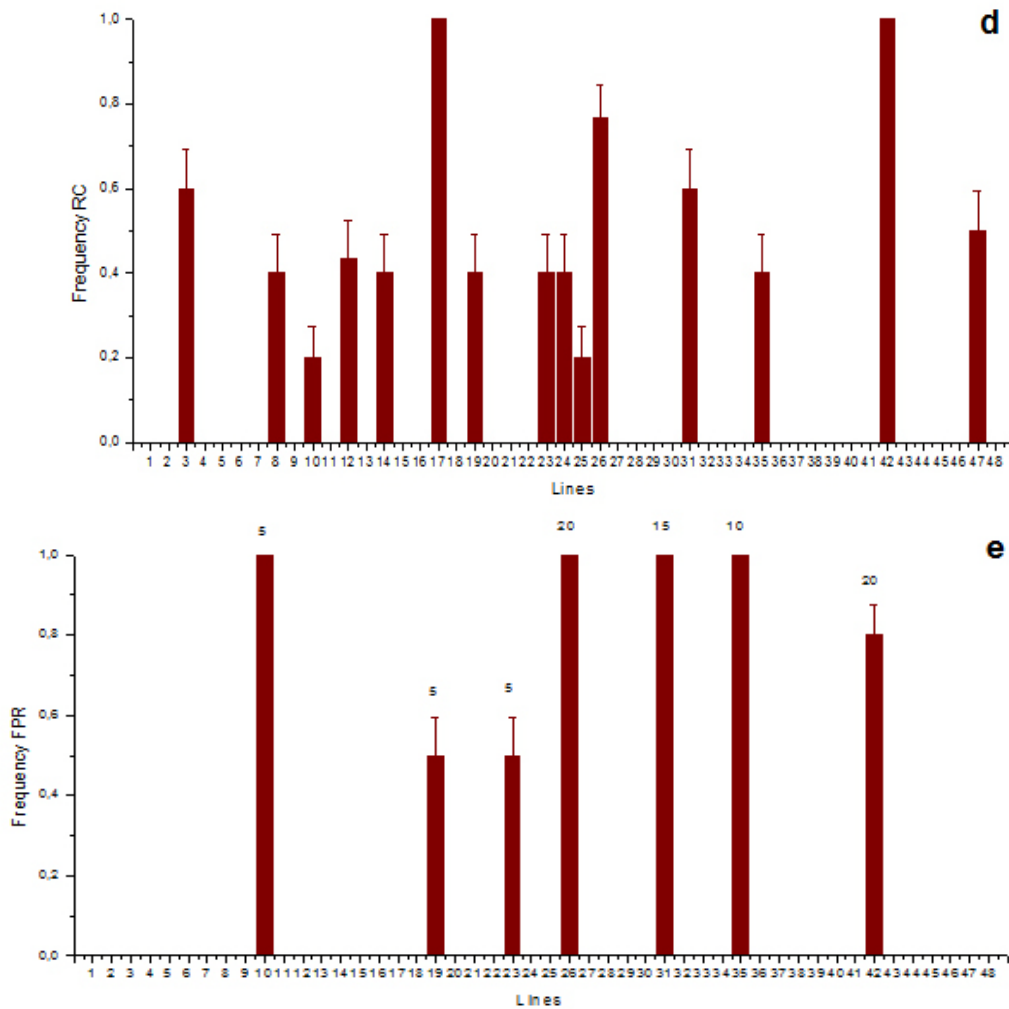
RESULTS

Callus induction

The response to *in vitro* culture of each line was recorded by the observation of each immature zygotic embryo. Considering the differences in the response to the *in vitro* culture observed between the lines, a Line Index (LI) was developed. This index was determined according to the following scale: 1 = without response; 2 = watery callus; 3 = compact, organogenic callus; 4 = low embryogenic structures; 5 = friable, low embryogenic structures; 6 = friable, highly embryogenic structures (type-II-like). An average LI was obtained for each line considering all the explants cultured. Categories between 1 and 3 were considered as non-embryogenic responses and those between 4 and 6 were considered as embryogenic responses. Figure 1 shows the different embryo responses to *in vitro* culture.
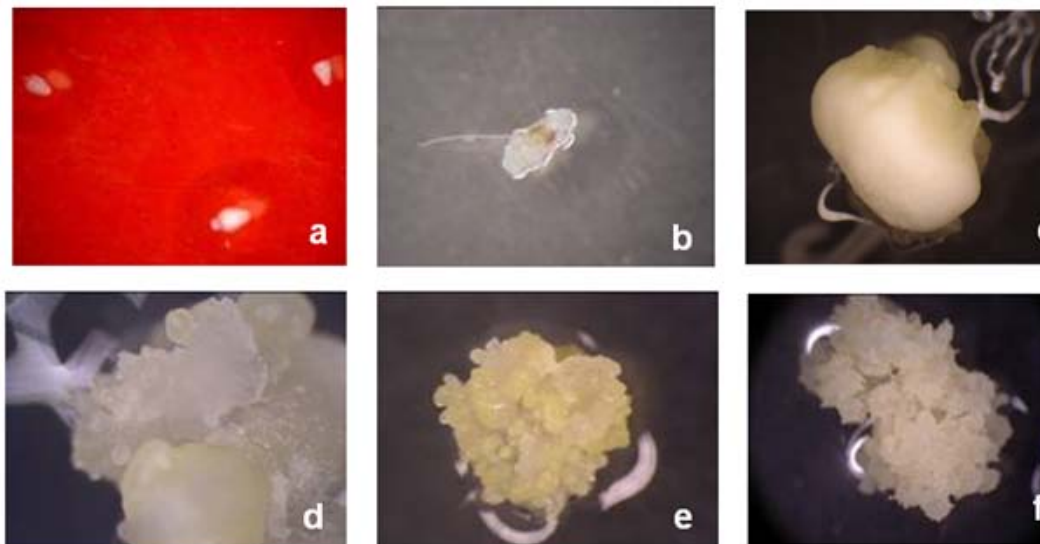
Fig. 1 Classification of different types of embryogenic responses. (a) type 1 = without response (Line nº 1); (b) type 2 = watery callus (Line nº 20); (c) type 3 = compact, organogenic callus (Line nº 47); (d) type 4 = low embryogenic structures (Line nº 19); (e) type 5 = highly embryogenic structures (Line nº 23); (f) type 6 = friable, embryogenic, type-II-like structures (Hi II).

Fig. 2 Results obtained of *in vitro* and *ex vitro* behavior of the 48 lines evaluated in the 2008-2009 maize growing season. (a) Line Index (LI) obtained by classification of callus type developed from each line. The parameters evaluated are expressed as frequencies; (b) *In Vitro* Capacity (IC); (c) Embryogenic Capacity (EC); (d) Regeneration Capacity (RC) and (e) Fertile Plants Recovery (FPR); the numbers of plants regenerated from each line are expressed on each bar.

The LI varied between 1.77 (line 1) and 4.36 (line 36). A total of 18 out of 48 lines showed an LI average above 3, while the remaining 30 lines showed an LI average below 3. Among the group of low LI lines, all the immature embryos had a uniformly poor response (showing lower variation as reflected by the standard deviation for each line), whereas in the high LI group the callus quality was not uniform (*i.e.*, it showed higher variation as reflected by the SD for each line) (Figure 2a). For example, line 23 had an average LI of 3.6, but the 41% callus ranked in category 5 (highly embryogenic, Figure 1e).

The *in vitro* capacity (IC) was calculated as the frequency of calluses with embryogenic response/total calluses formed. The results of the first growing season showed that 11 lines presented an IC higher than 0.4, 28 lines an IC lower than 0.4, and 10 lines did not have any embryogenic response (IC = 0) (Figure 2b).

Additionally, the embryogenic characteristic of calluses was assessed by histological cuts. The embryogenic calluses showed pro-embryogenic centers consisting of small differentially stained cells with prominent nuclei (Figure 3a), confirming the embryogenic character of the calluses analyzed.
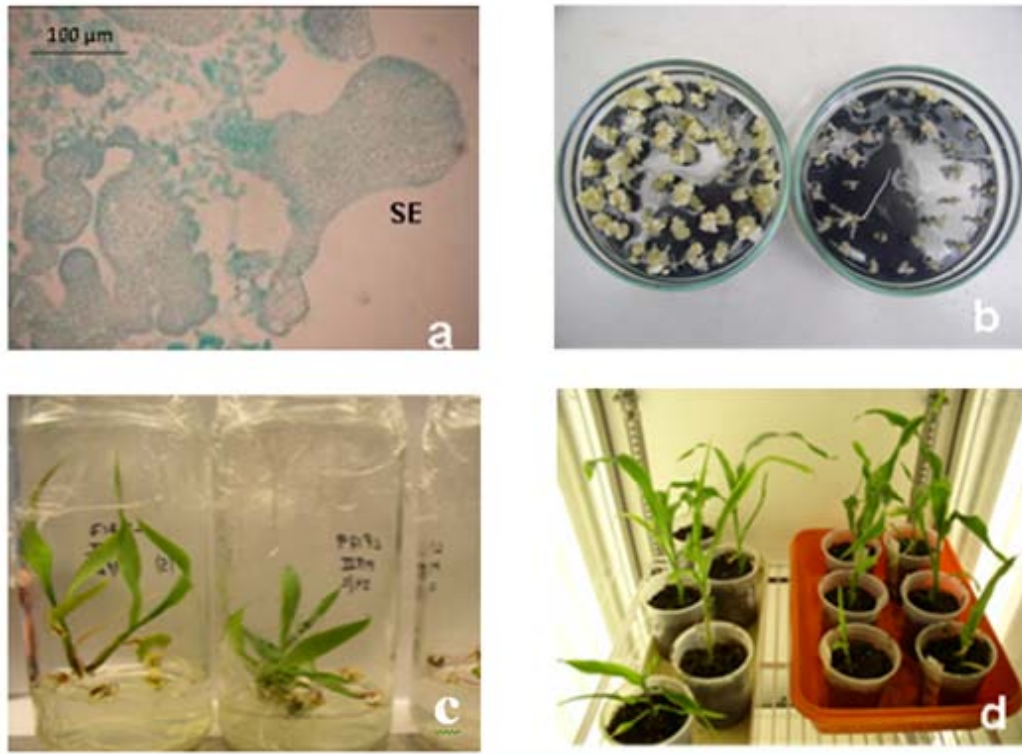
Fig. 3 Regeneration of Argentinean maize inbred lines. (a) Histological cut of an embryogenic callus, SE (somatic embryo in globular stage); (b) Differences between lines nº 19 (left) and 1 (right) in the embryo formation capacity at 20 days in somatic embryo medium; (c) Germinated somatic embryos of lines nº 23, 26 and 42 (from left to right). (d) Regenerated plants by somatic embryogenesis of lines nº 23, 26 and 42 during the acclimation period in chamber.

Somatic embryogenesis

The embryogenic capacity (EC) was determined as the frequency of calluses that showed development of mature somatic embryos. The capacity to develop mature somatic embryos varied broadly among the lines evaluated (between 0 and 1). Figure 3b shows the differences between the calluses of line nº 18 (EC = 0.93) and those of line nº 1 (EC = 0.03) at the same culture period in regeneration medium. Calluses with low embryogenic capacity also had lower growth capacity and became brownish after twenty days in culture.

Regarding the EC, for the 2008-2009 maize growing season, lines nº 8, 12, 14, 18, 23, 26, 29, 34, 38 and 41 showed the highest values (EC > 0.7) of mature somatic embryo development (Figure 2c).

Regeneration

Seedlings from 14 out of 48 lines were recovered by somatic embryogenesis. The regeneration capacity (RC) was determined as the frequency of somatic embryos germinated. The values of RC for the lines studied varied between 0.4 and 1 (Figure 2d). The somatic embryos obtained from these selected inbred lines germinated normally under the culture conditions used (Figure 3c).

Whole plants were obtained from seven genotypes. Lines n°26 and n°42 developed 20 plants each, whereas lines nº 10, 18, 23, 31 and 35 developed between 5 and 15 plants each starting from the culture of 30 mature somatic embryos (Figure 2e). All these regenerated plants were transferred to 5-L pots to the greenhouse for further development. All the whole plants obtained showed a normal

development, indicating that there were no somaclonal changes, at least at the phenotype level, during the *in vitro* culture under the conditions applied (Figure 3d). Regenerated plants continued their normal development in greenhouse conditions. This shows that the selected lines tolerate the *in vitro* process up to the regeneration and acclimation steps.

Seeds were harvested from lines nº 10, 18, 23, 26, 35 and 42, whereas line nº 31 did not develop seeds. Figure 4 shows a fertile plant regenerated from line 23 and the ears harvested from lines nº 19 and 26.


Fig. 4 Fertility of regenerated plants. (a) Fertile plant regenerated from line nº 23; (b) and (c) Ears harvested from regenerated plants of lines nº 19 (b) and 26 (c).

The lines which showed an IC or EC above 0.5 in the 2008-2009 growing season were selected for further studies in the following season. Thus, lines nº 18, 19, 23, 26, 36 and 42 were subjected to the whole *in vitro-ex vitro*-greenhouse development and evaluated using the same IC, EC and RC indexes.

In the 2009-2010 growing season, lines nº 18, 19, 23, 26, 36 and 42 were studied. No significant differences (p < = 0.05) in the IC values were found between both seasons for each of the lines evaluated (Figure 5a). The values for the EC index were similar in both seasons for lines nº 18, 19, 23 and 26, but showed significant differences (p < = 0.05) for lines nº 36 and 42 (Figure 5b). In addition, RC values were similar in both seasons for lines nº 18, 19, 23, 26 and 36, whereas they differed for line nº 42 (Figure 5c). Consequently, the same behaviour was found for FPR.

Genetic divergence analysis

The lines included in this study were selected based on their extreme response to *in vitro* culture. A total of seven lines (18, 19, 23, 26, 29, 36 and 42) were chosen for their good *in vitro* performance (IC or EC > 0.5, Figure 2b and Figure 2c). On the other hand, lines nº 1, 7, 11, 21, 28 and 48 were chosen for their poor *in vitro* performance (IC or EC < 0.2, Figure 2b and Figure 2c). Line Hi II was also included in the molecular variability analysis.
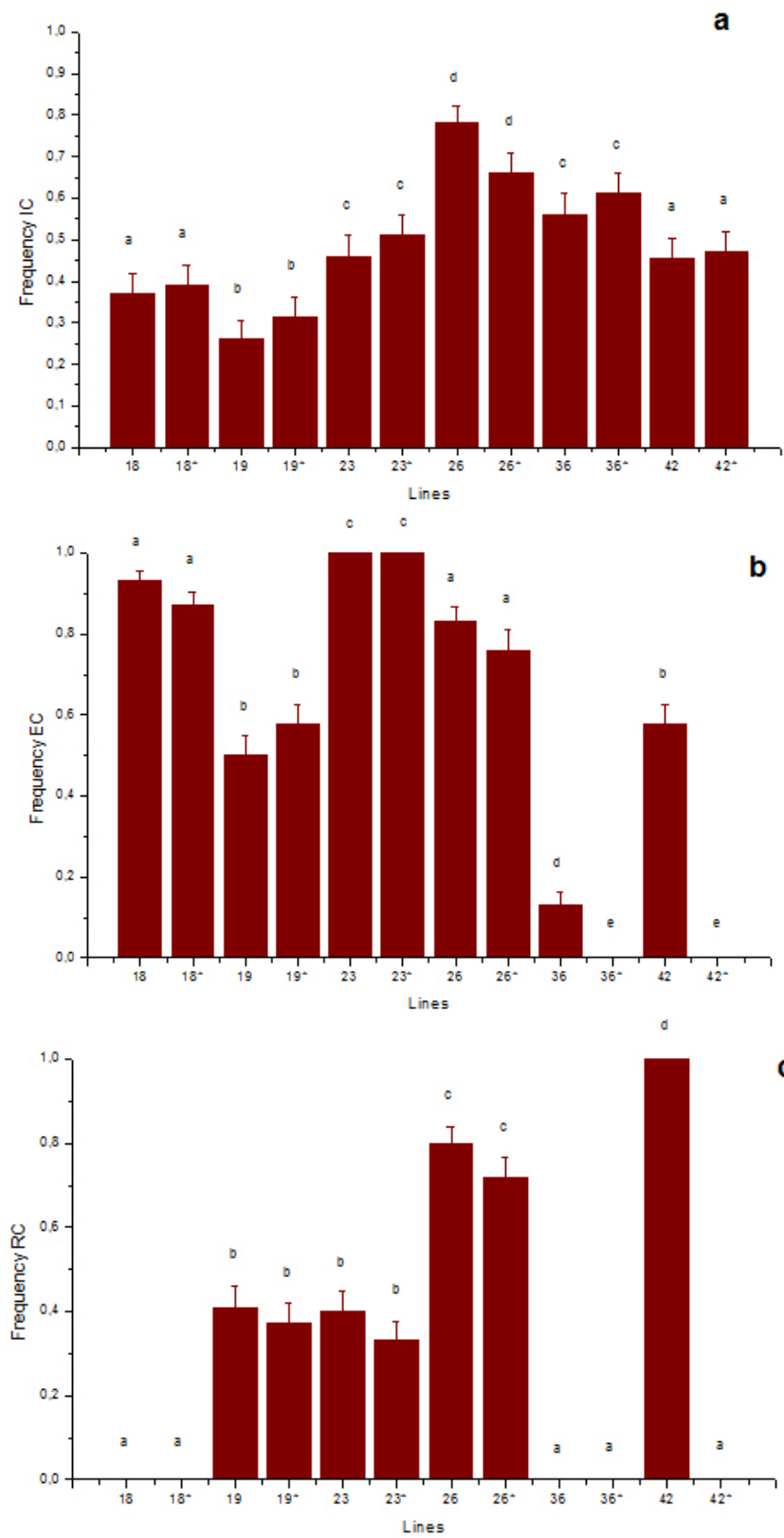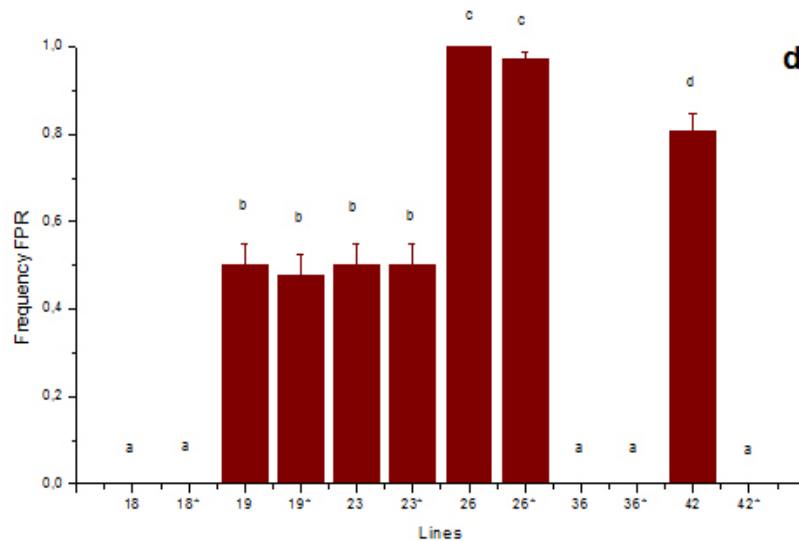
Fig. 5 Comparison between the 2008-2009 and 2009-2010 growing seasons. (a) *In Vitro* Capacity (IC); (b) Embryogenic Capacity (EC); (c) Regeneration Capacity (RC). Asterisks indicate the results of 2009-2010 for each line. Different letters indicate significant differences (p ≤ 0.05).

From the screening of 50 SSRs primers, 33 were selected upon their ability to produce stable and repeatable polymorphisms on polyacrylamide gels in the 14 inbred maize lines. Five primers failed to amplify products consistently and 12 primers were difficult to score accurately.

All the lines tested were molecularly differentiated using the 33 SSR markers.

Genetic Distances (GD) were calculated between the pairs of lines (GD = 1 – coefficient of similarity) and varied between 0.48 and 0.94 with an average of 0.84. Table 2 shows the GD between pairs of lines obtained with this group of microsatellite markers.

DISCUSSION

The capacity to produce regenerable calluses from immature embryos appears to be genotype-dependent in many species, including maize (El-Itriby et al. 2003; Che et al. 2006; Wang et al. 2009). Conventional transformation procedures in maize involve the use of genotype HI II, which has good characteristics for *in vitro* culture but poor adaptation to Argentinean agro-ecological conditions. In an effort to solve this problem, and to facilitate the integration of the new genes into a better background, it is strategic to evaluate the *in vitro* behaviour of Argentinean genotypes developed by breeding programs in public local institutions like INTA.

There have been several attempts worldwide to identify locally developed genotypes useful for *in vitro* culture and with good agronomic performance (Danson et al. 2006; Hernández-García et al. 2007; Binott et al. 2008; Anami et al. 2010). In the present study, we evaluated 48 inbred lines and identified seven lines capable of completing the whole cycle from the *in vitro* culture processes to the entire fertile plant, through somatic embryogenesis.

With respect to the genetic dependence of the *in vitro* response, surprisingly, almost all the 48 lines were capable of developing calluses but, at the somatic embryogenesis time, not all of them formed embryos. The line index (LI) was determined by observing the response to *in vitro* culture of each immature zygotic embryo. These exhaustive observations allowed us to classify the lines according to the type of calluses formed. In the lines with low LI (< 3), generally all the immature embryos had poor response, while in the lines with high LI (> 3), callus quality was not uniform (Figure 2a). These

11

observations suggest that, between the multiple factors influencing the *in vitro* response, the genetic background is crucial to obtain some differential behaviour among the lines tested. It is known that the induction of somatic embryogenesis must consist of the end of the current gene expression pattern in the explant tissue, and its replacement with an embryogenic gene expression program (von Arnold et al. 2002). This results in a series of cell divisions that induce either unorganized callus growth or polarized growth leading to somatic embryogenesis. The initiation of the embryogenesis pathway is restricted only to certain responsive cells in the primary explant which have the potential to activate the genes involved in the generation of embryogenic cells (Zhang et al. 2011). The competence for embryogenic induction may be the result of varying auxin sensitivity of these cells (von Arnold et al. 2002; Zhang et al. 2011). In this genetic context, the difference between the high and low LI lines could be attributed to differences in their capacity to activate the genes involved in the embryogenesis pathway. On the other hand, the heterogeneity for the LI values within the high LI line group could also be attributed to a heterogeneous pattern in the expression of the genes involved in this response. This could be explained by studying the expression patterns of the genes involved in the somatic embryogenesis pathway.

Table 2. Genetic distance calculated between pairs of lines included in the molecular assay variability.

	Hi II	23*	36*	42*	26*	29*	18*	11#	20#	19*	48#	28#	7#	1#
Hi II	0.00	-	-	-	-	-	-	-	-	-	-	-	-	-
23*	0.86	0.00	-	-	-	-	-	-	-	-	-	-	-	-
36*	0.90	0.76	0.00	-	-	-	-	-	-	-	-	-	-	-
42*	0.93	0.72	0.92	0.00	-	-	-	-	-	-	-	-	-	-
26*	0.86	0.89	0.89	0.92	0.00	-	-	-	-	-	-	-	-	-
29*	0.82	0.93	0.93	0.87	0.73	0.00	-	-	-	-	-	-	-	-
18*	0.64	0.76	0.61	0.96	0.81	0.81	0.00	-	-	-	-	-	-	-
11#	0.81	0.88	0.89	0.75	0.93	0.88	0.93	0.00	-	-	-	-	-	-
20#	0.87	0.93	0.78	0.88	0.81	0.81	0.78	0.93	0.00	-	-	-	-	-
19*	0.87	0.76	0.61	0.87	0.89	0.85	0.72	0.89	0.90	0.00	-	-	-	-
48#	0.80	0.94	0.87	0.97	0.73	0.94	0.87	0.85	0.79	0.90	0.00	-	-	-
28#	0.86	0.85	0.65	0.82	0.85	0.85	0.65	0.89	0.77	0.81	0.82	0.00	-	-
7#	0.79	0.93	0.94	0.83	0.86	0.90	0.86	0.85	0.79	0.94	0.85	0.90	0.00	-
1#	0.76	0.88	0.91	0.85	0.88	0.84	0.94	0.48	0.92	0.85	0.82	0.84	0.78	0.00

* good in vitro behaviour.
# bad in vitro behaviour.

In spite of the heterogeneity we were able to identify seven lines suitable for *in vitro* culture, somatic embryogenesis and regeneration (lines nº 10, 19, 23, 26, 31, 35 and 42). All of them passed through all the steps of *in vitro* culture until they developed whole fertile plants. Three of them (lines nº 19, 23 and 26) showed this good performance in both years.

In vitro capacity is a crucial parameter for the production of plants through somatic embryogenesis. However, our results suggest that not all the embryogenic calluses are capable of regenerating a whole

plant (Figure 2b, 2c, 2d and 2e). This observation is consistent with that found by El-Itriby et al. (2003), who concluded that the classification of calluses as embryogenic does not necessarily imply regenerability.

Results obtained in the 2009-2010 maize growing season suggest that calluses formed from lines nº 19, 23 and 26 can be maintained in culture for three months with subcultures every two weeks (Figure 5a). Moreover, they retain the ability to produce mature somatic embryos, seedlings and fertile plants during this culture period (Figure 5b, 5c and 5d). These features are typical of type II embryogenic calluses described in embryogenic genotypes currently used in maize genetic transformation (Wang et al. 2009). On the other hand, lines nº 36 and 42 showed a significant decrease in their EC values, indicating that the promising embryogenic calluses obtained from these lines were not capable of maintaining the embryogenic capacity during a culture period longer than one month, as observed in the first growing season. This feature is typical of type I embryogenic calluses (Wang et al. 2009). However, whole fertile plants were regenerated from lines nº 36 and 42 in the first year. This result is consistent with the findings of Bohorova et al. (1999) and El-Itriby et al. (2003), who reported that the regeneration could be obtained from both Type I and Type II calluses. It is important to highlight the possibility of maintaining the regeneration capacity for at least three months for some of the lines (lines nº 19, 23 and 26). The possibility to replace the Hi II genotypes with the new genotypes identified in the transformation procedures, as the *Agrobacterium* protocol proposed by Frame et al. (2011), remains to be proved.

Several *in vitro* culture parameters, such as age of immature zygotic embryo and concentrations and types of growth regulators, have been tested or adjusted elsewhere to obtain plants by somatic embryogenesis (Hernández-García et al. 2007; Binott et al. 2008; Anami et al. 2010). In the present study, constant *in vitro* culture conditions for the explants analyzed were maintained in order to choose the appropriate line that was able to adapt to the current transformation protocol conditions. Nevertheless, it would be interesting to evaluate the behaviour of the other promising lines, by the variation of some parameters involved in the *in vitro* culture protocol.

Some of the lines evaluated *in vitro* were also studied at the molecular level with the aim to compare their genetic background in terms of similarity or divergence. Despite the low number of loci evaluated on the SSR assays among the selected lines, we observed that there was high level of genetic divergence. Then, the *in vitro* response heterogeneity could be due to this wide genetic divergence found with the SSR assays (Table 2). In the present study, we also reported the usefulness of SSRs in the determination of genetic diversity among 14 lines with extreme response to *in vitro* culture. Some studies have revealed that there is strong correlation between genetic distance based on SSR data and grain yield/yield heterosis (Reif et al. 2003; Xu et al. 2004). In order to obtain hybrids with high heterosis, combining desirable *in vitro* response (for both parental lines) and different genetic backgrounds, pairs of lines were identified (Binott et al. 2008). The pairs that showed the highest genetic distance were: 42 x 23, 42 x 36, 42 x 26, 29 x 23, 36 x 29 and 19 x 42. These combinations could be used for crosses, expecting to form hybrids with good *in vitro* response and grain yield heterosis. These crosses have been done and are currently being evaluated.

On the other hand, the identification of divergent lines for *in vitro* culture behaviour (lines with CI or EC > 0.5 and lines with CI or EC < 0.2) will allow us to select parents to construct mapping populations with the aim to identify chromosome regions controlling somatic embryogenesis response.

Regarding the transformation process, lines nº 19, 23 and 26 were identified as the most appropriate to be tested on *Agrobacterium tumefaciens* or biolistic transformation protocols, because they have high levels of *in vitro* capacity and embryogenic capacity, and are capable of regenerating into complete and fertile plants. In addition, they are able to maintain embryogenic capacity during a long culture period. In addition, these three lines have good grain yield, good resistance to diseases, and pollination fitness (Table 1). These traits make them promising to go ahead with the crosses proposed. In summary, we were able to identify some lines that show both good response to *in vitro* culture requirements and good agronomic performance.

This work reports the first screening of Argentinean maize inbred lines for their response to *in vitro* culture. The use of the lines that showed good *in vitro* behaviour in a transformation protocol could facilitate the entire process of obtaining transgenic events to attain acceptable agronomic performance.

In this context, this is the first step to achieve a simpler scheme for the development of transgenic events using local genotypes.

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