Shortening of generation cycles in inbred lines of maize (*Zea mays* L.) through embryo rescue technique

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Abstract

Embryo rescue techniques have been used as an approach to raise hybrids from incompatible crosses. These techniques have also proved to be valuable tools for maize improvement, since they allow reducing the duration of the generation cycles for speed breeding. The aim of this study was to identify an efficient embryo rescue protocol to evaluate the response of maize (*Zea mays* L.) embryo culture and compare its generation time (seed to seed) with the generation time of plants obtained by mature seed germination. To this purpose, we evaluated the germination efficiency, *in vitro* protocol efficiency, and days to flowering of three advanced maize inbred lines developed at the National Institute of Agricultural Technology of Argentina. A greenhouse traditional strategy was used as control. The embryo rescue technique allowed obtaining nearly four generations per year compared to the two generations obtained in greenhouse. All the plants obtained by the embryo rescue technique were morphologically normal and fertile. The results confirmed the possibility of using embryo rescue strategies to significantly reduce the duration of the generation cycles in maize.

Introduction

In the last decade, maize breeding and research programs have incorporated several technologies to increase the efficiency of breeding processes and to reduce the whole time process. Among these technologies, those related to biotechnology such as molecular marker-assisted selection, genomic selection, and techniques to accelerate the generation time by embryo rescue have been the most adopted (Rizal et al., 2014; Heffner et al., 2010).

The technique known as embryo rescue refers to a series of *in vitro* steps that promote the development of an immature embryo in a viable plant. In this technique, appropriate culture media are used to replace the endosperm and thus allow the embryo to continue to grow (Reed, 2004). Embryo culture has been used for various purposes such as the development of interspecific hybrids (Hechanova et al. 2018, Sharma et al. 2017) through doubled haploid production (Santra et al. 2017, Warchoł et al. 2018).

Maize breeders occasionally carry out two generations per year, capitalizing the possibility of growing plants in contrasting latitudes (out of season, winter nursery). Although this is a valuable tool, applied mainly by companies, which allows accelerating the stabilization of lines or the backcrossing process, it is a high-cost technique. As an alternative, breeders grow maize plants in a greenhouse with suitable temperature and light supplementation, a technique that allows reaching up to 1.5 generations per year.

In this context, the implementation of the embryo rescue technique is an interesting tool to obtain more than two generations per year and reduce the time to reach the stabilization of lines. This technique is particularly interesting in the development of genetically modified plants, considering the need to stabilize transgenes to achieve homozygous lines before phenotypic evaluation. In this case, stabilization is reached by doubled haploid production or several self-pollination cycles inside a biosafety greenhouse.

The embryo rescue technique consists in extracting the immature embryos in the ear formation. The embryos must be sufficiently formed to possess the primordia of the apical and root meristems in conditions to respond to the stimuli to germinate (Fassio et al., 1998). Taking into account the stages of the reproductive phase of the maize (scale of Ritchie and Hanway, 1982), the immature embryos should be extracted at the beginning of the milk stage (stage R3), usually 18 to

22 days post-pollination, when the kernels begin to display final coloring and have reached 80% humidity. The physiological maturity of the grain occurs at stage R6. At this stage, kernel moisture is approximately 35 % and kernels have reached their maximum dry weight. Therefore, if an embryo rescue system is implemented at stage R3, it could be possible to move on to the next generation, avoiding stages R4, R5 and R6.

Regarding maize transformation protocols, somatic embryogenesis, which consists in obtaining embryogenic calluses derived from immature embryos, has been the most used in the last twenty years. In maize, the capacity to produce embryogenic and regenerable calluses from immature embryos is genotype-dependent (Che et al. 2006; Wang et al. 2009, Décima Oneto et al. 2011). Hi II is the genotype used in most maize transformation protocols because of its high regeneration capacity (Armstrong et al. 1991, Frame et al. 2000; Shou et al. 2004; Ghosh Biswas et al. 2006; Aluru et al. 2008). However, Hi II plants show poor agronomic aptitude, small ears and protandry.

Based on the above mentioned, researchers at the National Institute of Agricultural Technology (INTA) of Argentina have assessed the somatic embryogenic capacity of several Argentinean inbred lines developed in the maize breeding programs, and found that lines LP509, LP317 and LP4703 have suitable *in vitro* response and good agronomic performance (Gonzalez et al. 2012). These lines are thus promising to be used in transformation protocols and of interest to study their response to rescue embryos.

In this perspective, the aim of this study was to evaluate the potential of shortening the life-cycle of inbred maize by avoiding the requirements of seed maturation, generating plants from immature embryos under greenhouse conditions.

Materials and Methods

Inbred lines

Three maize inbred lines from the INTA breeding program were used: LP317, LP509 and LP4703. LP317 and LP509 have an intermediate crop cycle, whereas LP4703 has a long crop cycle. Plants were grown in a greenhouse at 28–30 °C/23–25 °C day/night air temperature and a 16-h photoperiod, in 12-l pots. Plants were self-pollinated and whole ears were collected 15-20 days after pollination. Developing grains were excised and surface-sterilized using a commercial bleach [sodium hypochlorite] solution (0.6%) with Tween-20 (1 ml/l) for 20 min. The sterilized grains were then rinsed five times with sterile deionized water.

In vitro culture

A total of 373 immature embryos (1, 2 and 3 mm long) were carefully isolated and placed in regeneration medium, containing MS basal medium with vitamins (4.3 g/l), sucrose (30 g/l), myo-inositol (100 mg/l) and phytagel (2.5 g/l), at pH 5.8. The embryos were incubated in a growth chamber (16-h photoperiod, 226 µE/m2, 26°C) until stage V3. All the plantlets were then transferred to pots containing a 3:1 soil:vermiculite mixture and grown in a growth chamber (16-h photoperiod, 226 µE/m2, 26 °C and 90% humidity) for hardening for 1 week. After that, plantlets were transferred to 12-l pots and grown in a greenhouse (16-h photoperiod, 650 µE/m2, 30-20 °C day/night temperature).

As control, mature seeds from the three lines were sown under the same conditions in the greenhouse.

Traits and Measuraments

Several factors of the *in vitro* culture were investigated to determine the global productivity of the system. The factors evaluated included: I) immature embryo size, II) germination efficiency (GE), determined as the number of germinated plants/number of embryos, III) hardening efficiency (HE), determined as the number of hardening plants/number of germinated plants) and IV) system efficiency (SE), determined as the number of hardening plants/number of embryos). To determine possible alterations as a consequence of *in vitro* culture, the mentioned factors were evaluated at two consecutive generations.

Evaluation of plant development

Days to flowering (measured as days from embryo extraction to stigma pollination) and height (cm) (measured from the soil to the panicle) were compared between plants from immature embryos (embryo rescue) and plants from mature grains, for each genotype. The plants were self-pollinated for the second round of embryo rescue.

Statistical analysis

The INFOSTAT software (Di Rienzo et al. 2012) was used for all statistical analyses. An ANOVA was performed in cases where the data had a normal distribution. The differences ($p < 0.05$) found after conducting a two-way ANOVA were analyzed by Tukey's test ($\alpha = 0.05$). In cases where the data did not show a normal distribution, a non-parametric analysis (Kruskal Wallis) was performed.

Results

Embryo, plantlet and plant development

The evaluation of the germination efficiency, GE, of immature embryos from sterilized spikes, considering their size at the time of excision, showed that 1-mm-long embryos were too young and did not germinate (Figure 1A), while 2- and 3-long embryos germinated properly and gave viable plants (Figure 1B). Plants from mature grains and plants from rescued immature embryos showed no differences in embryo development or seedling phenotype, in any of the genotypes evaluated (data not shown).

LP4703. The hardening plantlets were transferred to the greenhouse to obtain fertile plants (Figure 1E). The SE was 0.48 for LP317, 0.46 for LP509 and 0.36 for LP4703. In all cases, there were no significant differences between each of the factors evaluated (Figure 2).

Days to flowering (days from the date of embryo extraction until the date of stigma pollination) showed significant differences ($p<0.005$) between the plants from embryos and the plants from seeds for lines LP317 and LP509, but not for line LP4703 (Figure 3). These differences were on average 15.66 days for LP 317, 14.53 days for LP509 and 8.56 days for LP4703.

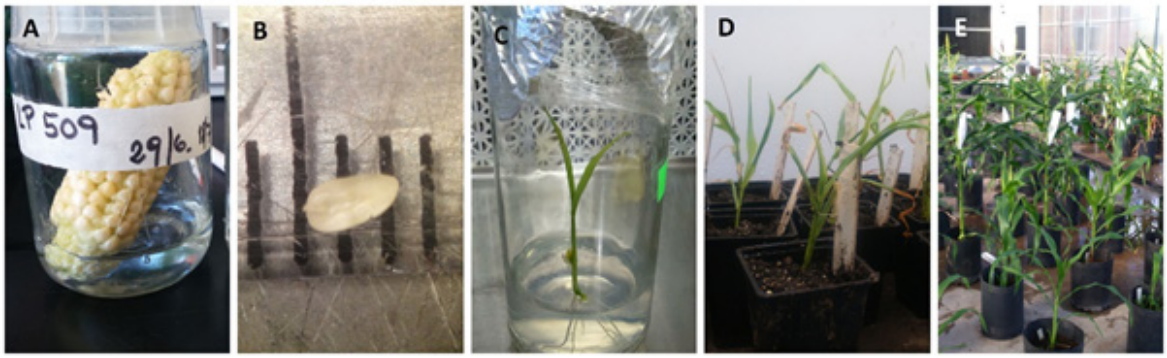

Fig. 1 - A: surface-sterilization of spikes, 20 days post-pollination. B: isolated immature embryos (2 mm long) of line LP509. C: plantlet *in vitro* from the embryo rescue technique. D: seedlings hardening in growth chamber. E: fertile plants from the embryo rescue technique in the greenhouse.

The embryo size at 15 – 20 days post-pollination was 2 mm. These embryos began to develop shoots and roots within 24 - 36 h of germination in plant regeneration medium (Figure 1C). The GE was 0.67 for LP317, 0.66 for LP509 and 0.53 for LP4703. At stage V3, plantlets were transferred to soil for hardening (Figure 1D). The HE was 0.72 for LP317, 0.70 for LP509 and 0.69 for

Height of plants in the greenhouse

The length from the plant's neck to the insertion of the panicle, the panicle length and the total height of the plant showed no significant differences between plants from rescued embryos and plants from seeds (Table 1). Despite the different growth conditions

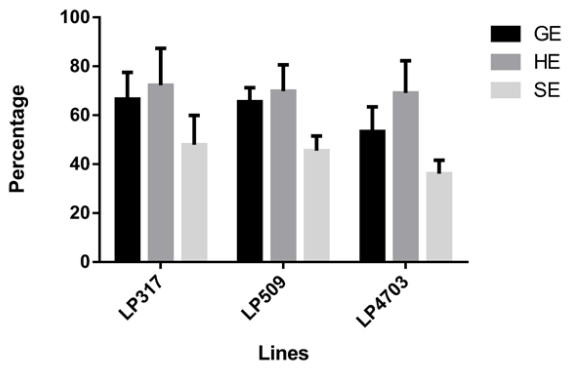

Fig. 2 - Germination efficiency (GE), hardening efficiency (HE) and system efficiency (SE) for the three maize lines under embryo rescue treatment. Error bars are represented.

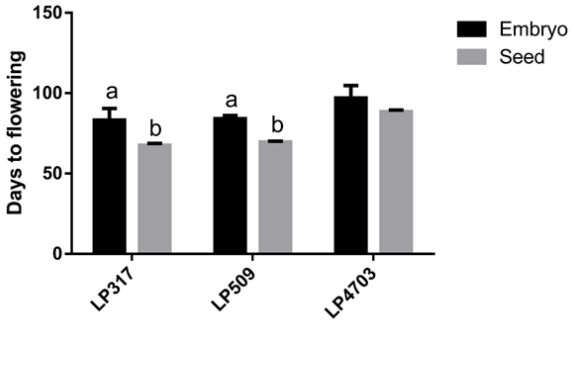

Fig. 3 - Days to flowering for the three maize lines under embryo rescue treatment and for those germinated from seeds. Different letters indicate significant differences ($p < 0.005$).

Table 1 - Length from the plant's neck to the insertion of the panicle, the panicle length and the total height of the plants from rescued embryos (Embryo) and plants from seeds (Seed) (mean ± s.d.).

Line	Length up to panicle (cm)	Panicle length (cm)	Total height (cm)
LP317 Embryo	79.15 ± 14.39	26.75 ± 6.36	105.90 ± 13.09
LP317 Seed	81.50 ± 9.60	29.25 ± 1.70	110.75 ± 9.03
LP509 Embryo	95.45 ± 18.64	33.45 ± 6.96	128.90 ± 23.15
LP509 Seed	88.60 ± 8.29	28.00 ± 2.00	116.60 ± 10.01
LP4703 Embryo	115.37 ± 20.51	20.75 ± 3.41	136.12 ± 21.83
LP4703 Seed	115.25 ± 11.59	20.25 ± 2.21	135.50 ± 13.72

(greenhouse vs. field), line LP4703 developed an average height close to that reported by the maize breeding Program of INTA, whereas lines LP317 and LP509 had lower height than those reported (Figure 4).

Total days per cycle and sum of cycles

The number of days from embryo extraction to the next embryo extraction was calculated by including in this period the extraction of immature embryos, germination in plant regeneration medium, hardening and transplant to the greenhouse, vegetative period, flowering, pollination and a new extraction of embryos. On average, this period was 103.33 days for LP317, 104.20 days for LP509 and 117.06 days for LP4307 (Table 2). Differences in this parameter were significant ($p<0.05$) only when LP317 and LP509 were compared with LP4307, thus showing that the differences are related to the length of the cycle.

In the present conditions (growth chamber and greenhouse), the long-cycle line (LP4307) can perform three cycles per year, while the two lines with shorter cycle (LP317 and LP509) can perform 3.5 cycles per year (Table 1).

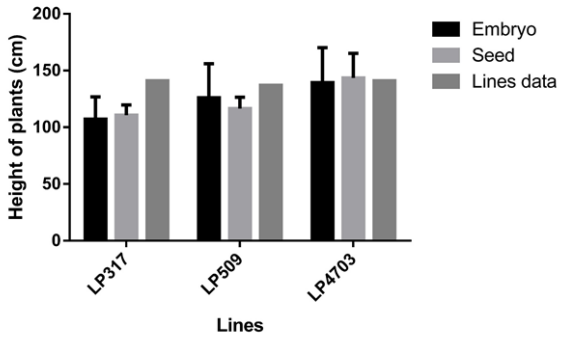

Fig. 4 - Total height of plants from seeds (Seed), from rescued embryos (Embryo) and data from breeding programs (Lines data) for the three inbred lines under study

Discussion

In addition to its agronomic importance, maize is a model crop for some topics of basic and applied research (Strable and Scanlon, 2009). In maize, as in other species, the capacity to produce regenerable calluses from immature embryos appears to be genotype-dependent (Che et al. 2006; Wang et al. 2009). Genotype HI II is widely used for conventional transformation procedures due to its good behavior in *in vitro* culture, but it has poor adaptation to Argentinean agro-ecological conditions. In the present study, we evaluated three inbred lines previously evaluated for their good somatic embryogenesis capacity (Gonzalez et al. 2012) and found that direct germination of immature embryos was possible.

Table 2 - Days from sowing to pollination and to produce embryos appropriate for the next cycle of in vitro culture, and cycles per year (mean ± s.d.).

Lines	Days to pollination	Days to embryo	Cycle per year
LP317	83.33 ± 7.22	103.33	3.53
LP509	84.20 ± 2.01	104.20	3.50
LP4703	97.06 ± 7.69	117.06	3.10

The method used was simple and reproducible and allowed the recovery of fertile plants from immature zygotic embryos from the three maize lines evaluated.

Lines LP317 and LP509 have an intermediate crop cycle, whereas LP4703 has a long one. This characteristic was maintained both in plants from rescued embryos and in plants from seeds grown in greenhouse and field conditions.

In this study, different comparisons between the maize lines and the *in vitro* culture conditions, through the embryo rescue cycles, were analyzed.

The size of embryos at 15 – 20 days post-pollination was 2 mm; the 5-day difference could be attributed to the environmental variations. Growth conditions may affect the interval (number of days) to obtain the correct size of embryos. Particularly, temperatures and solar radiation conditions (e.g. high-density canopy) could affect both embryo development and days to flowering (Andrade and Vega, 2000; Hammer *et al.*, 2009). In the greenhouse, the temperature and the light can be managed to supplement the deficiencies that can occur due to the shading by the ceilings and walls.

The parameter "flowering time" showed differences between plants from mature seeds and plants from rescued embryos, with the latter presenting a

delayed flowering time compared with the former. This was probably due to an insufficient temperature accumulation inside the growth chamber in relation to the greenhouse (Tollenaar et al., 2017; Lee et al., 2016). Other factors such as light intensity or culture medium itself may have influenced this parameter, but these factors were not studied individually in the present work.

The system efficiency was better in the lines with an intermediate cycle (LP317=0.48 and LP509=0.46) than in the line with long cycle (LP4703=0.36). However, these differences were not statistically significant.

The days to flowering parameter and total length of the cycle of line LP4703 showed significant differences. The results indicate that days to flowering and length of the cycle are parameters that should be taken into account when applying the embryo rescue technique in other lines (Joshi et al., 2016; Green and Phillips, 1975).

It is important to note that there were no significant differences in plant morphology between plants from seeds and plants from rescued embryos. This indicates that the *in vitro* culture *per se* did not alter the development of the plants. Nevertheless, differences were observed in the number of days to flowering: *in vitro* plants presented a delay in the days to flowering, in agreement with that observed by Dupuis and Dumas (1990).

The number of days per cycle of each genotype during culture of plants from immature rescued embryos showed an expected behavior according to the characteristics of each line (two of intermediate cycle and one of long cycle). The lines maintained their cycle length regardless of whether the plants came from seeds or from *in vitro* culture. This indicates that the cycle length is constant even when embryo rescue procedures are applied. This is in agreement with that reported by Ruiz Corral *et al*. (2002), who postulated that the main factors that determine the length of the cycle in maize are: i) the average number of days elapsed from sowing to physiological maturity and ii) the accumulated heat units either calculated with the classical residual method or using threshold temperatures.

Our results show that shortening the generations through the embryo rescue technique is feasible, at least in the maize lines studied. This technique may also be applicable for other maize lines with a similar cycle length, although parameters such as light intensity and temperature in the growth chamber and greenhouse should be adjusted to shorten the days to hardening.

Conclusions

This method significantly shortened the whole cycle length of maize inbred lines tested. This is especially important in transgenic maize research, because of economic and research reasons; shortening the generations is needed to advance generations in biosafety greenhouse and is useful for breeders who need to advance generations in a short time. The implementation of this system would allow obtaining up to 3.5 generations of maize line per year, reaching a number clearly higher than both, the two generations per year by winter nursery and the 1.5 generations per year through greenhouse with suitable temperature and light supplementation.

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