IN VITRO PROTOCOL FOR INDUCTION OF GAMETIC EMBRYOS FORMATION IN MAIZE (Zea mays L.)

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Abstract: The aim of the present study was to establish a protocol for the transformation of microspores and gametic embryos in maize. The response to isolated microspore culture of the three androgenic genotypes HKI-1011, HKI-1128 and HKI-1344 was studied. The response of the three genotypes to anther culture and the ability of their gametic embryos to undergo somatic embryogenesis and further regeneration of double haploid plants were also studied. The results revealed that the microspore culture approach results in a very poor androgenic response, despite the improvement achieved by adding activated charcoal (a max. of 43 embryos per 80000 microspores). The anther culture protocol, results in a high number of gametic embryos (a max. of 810 embryos per 100 anthers) that undergo somatic embryogenesis and regenerate into diploid plants at a high rate. For these reasons, preferentially gametic embryos from anther culture, rather than microspores, should be considered as targets for genetic transformation.

Key words: Gametic embryos, Maize

INTRODUCTION

Maize (Zea mays L.) is the third most planted cereal crop worldwide after wheat and rice. Globally, it is top ranking cereal in terms of productivity and has worldwide significance as human food, animal feed and fodder. It is used as a raw material for manufacture of large number of industrial products like corn starch and starch-based products, and in fermentation and distillation industries. Due to uses of maize and maize-based products, demand for maize is increasing across the world, and more predominantly in Asia [1].

Over the years, conventional breeding has been used as a tool for sustainable production of this crop. Nowadays, biotechnological tools can be helpful to enhance breeding and subsequently maize production. The in vitro androgenesis has been successfully applied to many species for the rapid development of fully homozygous transgenic lines. Green and Phillips [2] first reported that mature embryos of maize could be used to induce callus but no plantlets were regenerated. Wang [3] successfully regenerated plants from mature embryos of two maize inbreds, B73 and Mo17, but the regeneration was genotype dependent and the frequency was only 4 to 5%. Huang and Wei [4] reported regeneration of temperate maize lines from mature embryos at a frequency ranging from 19.85 to 32.4%. The ability to regenerate embryo derived from callus cultures has been reported to be dependent on the maize genotype used [5]. Most recently, Al-Abed et al. [6] reported more efficient regeneration system for two
hybrid and two inbred temperate maize lines using split mature seeds as an explant. So far almost all maize tissue culture and transformation involves the use of immature zygotic embryos as an explants source for regeneration [7,8].

The aim of the present experiment was to improve the existing protocols for the production of the explants used as targets for transformation (induced microspores and gametic embryos) and to determine which type of explant is more suitable as a target for transformation, based on their \textit{in vitro} performance.

**MATERIALS AND METHODS**

In this study genotypes were selected for androgenic responsiveness at the Biotechnology Laboratory, Department of Biological Sciences, RDVV, Jabalpur, Madhya Pradesh. These genotypes are HKI-1011, HKI-1128 and HKI-1344. The public line HM-10 was used as negative control, due to non-responsiveness to androgenesis. The donor plants were grown in the greenhouse at 28 °C (day) and 16 °C (night), a photoperiod of 15 hrs and a light intensity of 220 \text{ìmol m}^{-2} \text{s}^{-1}.

**In vitro Androgenesis:**

**Tassel harvest and cold stress treatment:** Tassels were removed from the plants wrapped in their leaf whorl with microspores at an early bi-nucleated developmental stage (75 days after sowing) and wrapped in moist paper towels and aluminium foil. The material was kept at 10 °C for two weeks for a cold stress treatment.

**Sterilisation of the spikelets:** The spikelets were removed from the main branch of the tassel and put into a 100 ml tube and 60 ml of a 2.5 \% (w/v) sodium hypochlorite solution, supplemented with 0.1 \% (v/v) detergent were added. The tube was rocked gently for 20 min; the spikelets were rinsed three times in sterile, double distilled water.

**Microspore culture (sucrose pulse method):** Sterile spikelets were pre-cultured in a 100 ml tube containing 60 ml of cold pre-culture medium for 72 hours at 16 °C in the dark. Then, the spikelets in the pre-culture medium were transferred to the container of a Waring blender (250 ml), which was sealed with aluminium foil. Two pulses of 12 s each at speed 3 (12600 rpm) were applied to release the microspores. The slurry was passed through a 120 \text{ìm} sieve, and the filtrate was collected in a 100 ml tube. After centrifugation at 180 \times \text{g} for 5 min, the supernatant was discarded and 60 ml of cold induction medium sucrose IMS were added. This sucrose pulse was applied for 15 min. Thereafter, three washes with induction medium maltose (IMM) were performed. The final density was adjusted to 50,000 microspores per ml IMM. Two ml of the suspension was put into each Petri-dish, 2.5 cm diameter. The protocol was varied by adding six small cubes (0.25 cm x 0.25 cm x 0.25 cm) of IMM (lacking TIBA) supplemented with 10 g/l activated charcoal and solidified with 10 g/l phytagel to each petri-dish. The cultures were kept at 28 °C in the dark for 30 days until the developing gametic embryos reached a diameter of 1.0 to 1.5 mm. Seven repetitions of ten petri-dishes each were performed for each genotype and protocol (with and without the addition of AC to the cultures).

**Anther culture:** The three anthers in the larger floret of the spikelet were isolated using forceps under sterile conditions. Twenty-five anthers were placed in petri-dishes containing 10 ml of induction medium liquid IML supplemented with 250 mg/l colchicine and cultivated for one week at 15 °C in the dark. Thereafter, the anthers were transferred to Petri-dishes containing induction medium semi solid IMSS and cultivated for one month in the dark at 27 °C until the emergence of the gametic embryos [9].

**Culture of immature zygotic embryos:** Ears of self-fertilized HKI-1128 plants were harvested 15 days after pollination and kept at 4 °C for 5 days. The kernels were separated from the cob and sterilized in a 2.5 \% (w/v) sodium hypochlorite solution supplemented with 0.1 \% (v/v) detergent for 15 min. The kernels were rinsed three times in sterile double distilled water. The embryos (1.5 to 2.0 mm in length) were isolated with a scalpel under sterile conditions and transferred to medium for secondary embryogenesis.

**Secondary embryogenesis:** Secondary embryos were induced from gametic and immature zygotic embryos on modified $N_6$ medium supplemented with 1 mg/l 2, 4-D. The zygotic embryos were placed on the medium with the scutellar side up. The cultures were kept in the dark at 28 °C and sub-cultured onto fresh medium at 15 days intervals.
Plant regeneration: Secondary embryos of zygotic and gametic origin (in the case of an indirect regeneration system) or embryos directly explanted from microspore or anther culture (in the case of a direct regeneration system) were placed in Petri-dishes, 15 cm in diameter, containing autoclaved Sigma MS medium [10] supplemented with 3 % (w/v) sucrose and solidified with 3 mg/l phytagel; the pH was adjusted to 5.7. The cultures were maintained at 28 °C in constant light (100 μmol m⁻² s⁻¹). The cultures were transferred to fresh medium every 15 days and the regenerating shoots were subcultured to magenta boxes containing the same medium. Plantlets of 8 cm were transferred to soil and covered with a translucent plastic beaker for 7 days to enable the leaves to adapt to dryer conditions. When possible, plants of gametic embryo origin were self-pollinated in order to establish a DH line.

Determination of the ploidy level: The ploidy analyser I was used to determine the ploidy level of regenerated shoots. Using a sharp razorblade, a leaf segment of approximately 10 mm² was cut into small pieces in 5 ml of cold (6 °C) DAPI staining solution and passed through a nylon gauze (45 μm mesh size). The filtrate was used for flow cytometric analysis; at a par gain FL₁ of 410 to 425 (relative fluorescence), a peak set at 150 and 250 FL (for G₁- and G₂- or M-phases, respectively) was interpreted as corresponding to diploid or double haploid material. A peak set at 75 and 125 FL was interpreted as corresponding to haploid material.

RESULTS

Microspore culture: The androgenic genotypes HKI-1011, HKI-1128 and HKI-1344 and the non-androgenic negative control line HM-10 were tested for their response to isolated microspore culture. A simplified version of a protocol developed by Nägeli et al. [11] and a variation of it, which involved the addition of AC to the induction medium, were used. When cultured on medium without AC, the androgenic genotypes HKI-1011 and HKI-1128 and the control genotype HM-10 did not respond to the microspore culture (Table 1). Only the genotype HKI-1344 yielded an average of 6 and a maximum of 43 gametic embryos per Petri-dish for the genotypes HKI-1344, HKI-1011 and HKI-1128, respectively (Table 1). The control line HM-10 did not show any androgenic response. The response to the androgenic induction differed significantly (p = 0.05) with and without the addition of AC to the culture medium, as depicted for the genotype HKI-1344. A clear morphological difference was determined between the gametic embryos developed in the presence or absence of AC. Whereas, the embryos developed in AC were compact and had a defined morphology, grown on medium without AC consisted mainly of a soft and brownish clump of undifferentiated cells. Due to the significantly stronger response of the genotype HKI-1344 to the microspore culture, it was selected for further experiments on plant regeneration.

HKI-1344 gametic embryos generated in medium with and without AC were subjected to regeneration through a direct and an indirect regeneration pathway: embryos were either transferred immediately after isolation to MS medium or cultivated for 28 days on modified N6 medium supplemented with 2, 4-D for the induction of secondary embryogenesis, prior to regeneration on MS. Five repetitions were performed per treatment (20 embryos per repetition). Whereas the direct regeneration method yielded 0.06 vs. 0.01 regenerants per embryo developed on medium without and with AC, respectively, the indirect regeneration method yielded 0.45 and 0.30 regenerants (Table 2). Despite the clear differences in the regeneration ability of the gametic embryos developed with and without AC, the yields did not differ significantly at p = 0.05. Significant differences were determined between the direct and indirect regeneration pathways. The regenerated plants were analyzed for their spontaneous diplodisation rate. From almost 100 tested individuals, 30.10 % showed a doubled chromosome number.

Anther culture: 5400 anthers of HKI-1011, 6000 of HKI-1128, 1500 of HKI-1344 and 1400 of HM-10 were isolated and subjected to conditions that induce gametic embryogenesis. Although a very high standard deviation was generally observed in these experiments, androgenic responsiveness differed significantly among all the tested genotypes. With an average of 309 and a maximum yield of 810 embryos per 100 isolated anthers, HKI-1344 was superior to the other genotypes, while the control line HM-10 did not respond to androgenesis (Table 3).
Fig. 1  Secondary embryogenesis. Somatic embryogenesis from zygotic genotype HKI 1128.

Fig. 2  Secondary embryogenesis. Somatic embryogenesis from gametic genotype HKI 1011. Embryos was induced by cultivating the embryos on Modified N₆ medium supplemented with 2,4-D. The callus structures correspond to the callus Type I.
The ability of the gametic embryos of HKI-1011, HKI-1128 and HKI-1344 to undergo secondary embryogenesis was assessed for eight weeks and compared to the induction of secondary embryogenesis of zygotic embryos of the genotype HKI-1128. Four weeks after the induction on Modified N6 medium containing 2, 4-D, the callus obtained from zygotic embryos showed structures corresponding to the Type I callus. The gametic embryos yielded secondary structures resembling those developed from zygotic embryos. During the 56 days period, an exponential proliferation of secondary embryos was observed in all cases. A significantly stronger response was found for the zygotic than for the average gametic embryos. Within the gametic embryos, no significant differences were found. After 28 days of culture on Modified N6 medium, the zygotic embryos yielded an average of 12 secondary embryos per explanted embryo, whereas the mean of the gametic embryos was five secondary embryos per explanted embryo. After eight weeks, the zygotic embryos yielded 69 and the gametic embryos 16 secondary embryos per explanted embryo, indicating a five-fold stronger response of the zygotic embryos. Furthermore, the ability of the secondary embryos to regenerate into a plant, depending on the duration of their exposure to N6 medium containing 2, 4-D, was studied. Secondary embryos of zygotic and gametic origin were transferred to regeneration medium after 2, 4, 6 and 8 weeks of cultivation on N6 medium and the total number of regenerating shoots was determined. Compared to the secondary embryos of gametic origin, those of zygotic origin showed a superior response and yielded 0.5 regenerants per secondary embryo, when they were two to four weeks old. With increasing age, there was a sudden decrease in the number of regenerants per secondary embryo. Secondary embryos of gametic origin yielded 0.11 regenerants per secondary embryo when they were 15 to 30 days old and an average of 0.06 regenerants per secondary embryo when they were 50 days old. No significant differences were found for the secondary embryos derived from gametic embryos. In all cases, the potential of secondary embryos to regenerate clearly decreased with increasing cultivation time on N6 medium. The final yield of regenerated shoots per explanted embryo increased linearly with increasing cultivation time on N6 medium for all genotypes. The yield of regenerants per zygotic embryo was always significantly higher than the yield per gametic embryo.

**Table 1:** Response to microspore culture of four genotypes and effect of AC. The number of embryos per 80 000 microspores grown in medium without and with AC (IMM - AC and IMM + AC, respectively) shown. Data followed by the same letter were not significantly different from each other at p = 0.05.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Genotypes selected for androgenic response</th>
<th>Control genotype HM-10</th>
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<tbody>
<tr>
<td></td>
<td>Genotypes selected for androgenic response</td>
<td>Control genotype HM-10</td>
</tr>
<tr>
<td>IMM - AC</td>
<td>HKI-1344 5.8 ± 7.4</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>IMM + AC</td>
<td>HKI-1011 0 ± 0 a</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>IMM + AC</td>
<td>HKI-1128 0 ± 0 a</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>IMM + AC</td>
<td>HKI-1344 0 ± 0 a</td>
<td>0 ± 0</td>
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**Table 2:** Regeneration ability of microspore culture derived embryos. Embryos developed in IMM with and without AC were subjected to a direct and an indirect regeneration pathway. Data followed by the same letter were not significantly different from each other at p = 0.05.

<table>
<thead>
<tr>
<th>Regenerants per embryo developed without AC</th>
<th>Direct regeneration</th>
<th>Indirect regeneration</th>
</tr>
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<tr>
<td>0.06 ± 0.07 a</td>
<td>0.45 ± 0.28 b</td>
<td></td>
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<table>
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<tr>
<th>Regenerants per embryo developed with AC</th>
<th>Direct regeneration</th>
<th>Indirect regeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01 ± 0.02 a</td>
<td>0.30 ± 0.26 b</td>
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**Table 3:** Androgenic response and diploidisation rate. Three ETH genotypes (selected for androgenic response) and the control genotype HM-10 were compared with regard to androgenic response and rate of diploidisation. Data followed by the same letter were not significantly different from each other at p = 0.05.

<table>
<thead>
<tr>
<th>Genotypes selected for androgenic response</th>
<th>Control genotype HM-10</th>
</tr>
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<tr>
<td>HKI-1011 85.6 ± 75.6</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>HKI-1128 39.2 ± 51.2</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>HKI-1344 309.3 ± 263.5</td>
<td>0.0 ± 0.0</td>
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HKI-1128 and HKI-1344, was determined (Table 2). On average, 54% of the individuals were double haploids. The genotype HKI-1128 tended to have a higher rate of diploidisation (63%) than HKI-1344 (55%) and HKI-1128 (42%).

**DISCUSSION**

**Microspore culture:** The simplified microspore culture protocol was adopted to test three androgenic HKI genotypes and the non-androgenic control line HM-10 [11]. As expected, the negative control line did not respond. The androgenic response of the genotypes HKI-1344, HKI-1011 and HKI-1128 was very low compared to the yields achieved by Nägeli et al. [11]. This might be due to the use of a simplified version of their protocol, which lacks a gradient separation of the microspores that enables the selection of qualitatively better microspores but, most probably, due to the use of other genotypes. The addition of activated charcoal to the cultures led to an almost 6-fold increase in yield in the case of the genotype HKI-1344. A notable difference was found in the morphology of the embryos developed in the presence or absence of activated charcoal. Whereas embryos developed with AC showed differentiated structures, those devoid of AC had a highly undifferentiated morphology and were brown in colour, attributable to the synthesis of phenolic compounds [12]. The higher levels of differentiation observed for the former embryos may be due to the absorption of the phenolic compounds and other substances inhibiting both growth and differentiation by the activated charcoal. For example, the Group of Agronomy and Plant Breeding of the HKI demonstrated that AC plays a role in the gradual absorbance of the exogenous hormone TIBA. It is interesting that this substance, which inhibits auxin transport [13], is essential for the induction of androgenesis but stops it shortly thereafter when not removed from the medium.

The different morphology of both types of embryo had an effect on their ability to undergo somatic embryogenesis: when transferred to N6 medium containing 2, 4-D, the brown coloration of the embryos disappeared shortly thereafter and cell division was reactivated. A high ability to undergo callus formation and subsequent secondary embryogenesis was observed, a phenomenon attributable to the higher proportion of undifferentiated cells; this factor probably simplified the reactivation of cell differentiation.

An extremely high regeneration rate of 0.45 regenerants per embryo was achieved. On the other hand, embryos developed on medium with activated charcoal did not multiply at such a high rate, leading to a lower output of regenerants (0.30 regenerants per embryo). These differences between both types of embryo were, however, insignificant due to a very high variability between repetitions, which led to extremely high standard deviations. A highly significant difference was determined between the yields obtained by the direct and the indirect regeneration pathways. The indirect pathway resulted in a 10 times stronger response with regard to regenerants per embryo, yielding a mean of 0.38 regenerants per embryo. This yield is significantly higher than that reported by [11,14,15]. A spontaneous doubling efficiency of 30% was observed, which is higher than the doubling efficiency reported by Nägeli et al. [11]. Despite the improved response of the HKI-1344 genotype to the microspore culture and an excellent rate of plant regeneration by means of the indirect regeneration pathway, the procedure is not sufficiently efficient for the satisfactory production of gametic embryos and double haploid plants. The results clearly showed that microspores are hardly able to develop without the tapetal cells of the anthers. Even extremely responsive genotypes like HKI-1344 do not produce the number of gametic embryos required for further applications. This drastically diminishes the chance of achieving regenerants from genetically transformed microspores.

**Anther culture:** The response to anther culture, i.e. the ability of microspores to form gametic embryos in anthero, was studied for the three highly androgenic genotypes (HKI-1011, HKI-1128 and HKI-1344): whereas the control line HM-10 showed no response, HKI-1344 exhibited the highest yields ever observed [15,16]. HKI-1011 and HKI-1128 also provided a constant supply of gametic embryos. The high standard deviation in androgenic responsiveness is explained by i) the extreme sensitivity of the system to variations in environmental conditions, ii) the developmental stage of the donor material and iii) the segregation of traits that influence androgenic responsiveness. A mean diploidisation rate of 55% was achieved as a result of the colchicine treatment, in agreement with the results of Nägeli et al. [11]. Armstrong and Green [17] described two types of callus (I and II) which can originate from the cultivation of immature zygotic embryos on medium.
containing 2,4-D. In general, Type I callus is white and compact and seems to be more differentiated than the Type II callus. The latter is soft, can retain totipotency after longer periods in culture than the Type I callus, but its establishment is difficult and highly genotype-dependent. The culture of HKI-1128 zygotic embryos on the Modified N6 medium induced Type I callus only, which was found to be suitable for transformation [18]. When HKI-1011, HKI-1128 and HKI-1344 gametic embryos were cultured on the same medium, structures resembling the Type I callus were obtained, indicating that this explant might be as suitable for transformation experiments as the zygotic embryos. The number of secondary embryos of zygotic and gametic origin multiplied exponentially but rapidly lost their ability to regenerate after four weeks. This is typical of the Type I callus and was proposed by Jiménez and Banghert [19] to correlate with endogenous IAA levels. The ability of zygotic embryos to undergo secondary embryogenesis after four weeks of culture on modified N6 medium was five-fold lower than for zygotic embryos. The cells of gametic embryos do not undergo somatic embryogenesis to the same extent as the scutellar cells of the zygotic embryos [20].

The combination of an exponential multiplication of secondary embryos and a reduction in their ability to regenerate into a plant resulted in an increase in the number of regenerants per original zygotic and gametic embryo during the eight weeks of the experiment. Rates of 8.2 and 1.0 regenerants per original embryo were achieved for eight-week-old zygotic and gametic embryos, respectively. Saisingtong et al.[9] and Büter et al.[21], who applied a direct regeneration procedure, reported a maximum of 0.2 regenerants per gametic embryo. It is expected that, after a prolonged culture on N6 medium, regenerants would no longer form because of the age-correlated decrease in the regeneration ability of secondary embryos, which overcompensates the proliferation achieved by secondary embryogenesis.

**CONCLUSION**

The gametic embryos were responsive to the induction of secondary embryogenesis and plant regeneration from secondary embryos. Despite, the zygotic control was much more efficient than the gametic embryos with regard to all the studied factors, the results reveal that the gametic embryos have the characteristics required for their use as a target for genetic transformation, which may be further useful for the development of homozygous and transgenic maize lines.

**REFERENCES**


