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Embryo rescue and induction of somatic embryogenesis as a method to overcome seed inviability in *Zea mays* ssp. *mays* × *Zea mays* ssp. *parviglumis* crosses

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Abstract

Zea mays ssp. *mays* (2n=40) and *Z. mays* ssp. *parviglumis* (2n=20) were crossed to obtain hybrid plants by embryo rescue. Hybrid embryos were isolated and cultured on García *et al.* (1992) basic medium supplemented with 2,4-dichlorophenoxyacetic acid and/or kinetin in different concentrations. Caryopses harvested 23 d after pollination (DAP) were turgid, with 0.3 to 0.5 mm long embryos, while those harvested 30 DAP were shrunken, with 1 to 1.5 mm long embryos. Twenty days after plating, 100 % of the younger embryos gave rise to white, compact embryogenic calli. Subsequently, coleoptiles, leaf-like structures, shoots and roots originated from them and 35 hybrid plants were regenerated from 60 embryos. Embryogenic or organogenic calli frequencies did not differ among hormonal treatments, but they decreased, on average, from 90.5 to 44.3 %, comparing 50 and 120-d-old cultures. The older embryos regenerated plants only by germination, although they gave rise to organogenic callus with low frequencies. Regenerated plants showed a somatic chromosome number of 2n=30, pollen fertility of 40 to 80 % and 15 % viable naked caryopses.

Additional key words: 2,4-dichlorophenoxyacetic acid, incompatible crosses, maize hybrids, organogenesis, plant regeneration, tissue culture.

Introduction

The genus *Zea* comprises two perennial species, *Z. perennis* (2n=40) and *Z. diploperennis* (2n=20), and one annual species, *Z. mays* (2n=20), which embraces three subspecies: *mexicana*, *parviglumis* and *mays* (Doebley and Iltis 1980, Doebley 1990). Therefore, interspecific crosses within the genus *Zea* result in hybrids with somatic chromosome numbers of 2n=20, 2n=30 or 2n=40. Further, hybrids with somatic chromosome numbers 2n=20, 30 or 40 can also be obtained from crosses between colchicine-duplicated maize inbreds (2n=40) and some other *Zea* species. The 2n=20 and 2n=40 hybrids (balanced gametes) showed fertile pollen and viable seeds, while 2n=30 hybrids (unbalanced gametes) were highly sterile (Molina and García 1999).

Embryo rescue has been useful for plant breeding purposes as well as to elucidate evolutionary relationships in the genus *Zea*. This method allowed to obtain plants of *1*) incompatible interspecific and intergeneric hybrids of maize and its wild relatives, such as *Z. mays* × *Z. perennis* (Molina and García 1999) and *Tripsacum* × *Zea* (Farquharson 1957, García and Molina 1999), *2*) haploid wheat from *Triticum* × *Zea* crosses (Laurie and Bennett 1988), *3*) defective endosperm mutants (Sheridan *et al.* 1978), and *4*) triploid maize (García and Molina 1995). Although many researchers regenerated maize plants by somatic embryogenesis and organogenesis from immature embryos (*e.g.*, Green and Phillips 1975, Lu *et al.* 1982, Van Lammeren 1988), there are few reports about plant regeneration by organogenesis or somatic embryogenesis from maize and its wild relatives hybrids

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; DAP - days after pollination.
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(Furini and Jewell 1995, García and Molina 1997). The efficient use of this technique makes it possible to produce many plants per embryo, which could notably increase the number of regenerated hybrid plants.

The purpose of this work was to regenerate plants by organogenesis or somatic embryogenesis from *Z. mays* ssp. *mays* (2n=40) × *Z. mays* ssp. *parviglumis* hybrid embryos (2n=30).

Materials and methods

Two inbred lines of *Z. mays* ssp. *mays* 2n=40 (N107B and N103A, supplied by Maize Genetics Cooperation Stock Centre, Urbana, IL, USA) and *Z. mays* ssp. *parviglumis* 2n=20 were grown in the greenhouse and hand pollinated during summer and autumn of 1996. *Z. mays* ssp. *mays* × *Z. mays* ssp. *parviglumis* (MPa30) hybrid ears were harvested 21, 23 and 30 d after pollination (DAP). Developed caryopses were surface sterilised with 2.5 % sodium hypochlorite. Embryos were excised, placed on the culture media and incubated at 28 - 30 °C in darkness for a month. Later, they were grown under a 16-h photoperiod, with an irradiance of 31 μmol m^{-2} s^{-1} and the same temperature as before. The culture media consisted of the García *et al.* (1992) basic medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin in different concentrations [μmol dm^{-3})]: 1 - 0 (D1); 1 - 0.5 (D1C); 2 - 0 (D2); 2 - 0.5 (D2C). Shoots produced on initiation medium were rooted on the basic medium free of plant growth regulators, under the same conditions of irradiance and temperature. Regenerated plants were rusticated in pots with soil and a plastic cover, 3 weeks later, plants were transplanted to soil and grown in a greenhouse. Frequencies were analysed with the Brant and Snedecor test (Cochran and Cox 1965).

Results

Inbred lines N107B and N103A pollinated with *Z. mays* ssp. *parviglumis* showed the development of caryopses in the whole ear. Up to 23 DAP they were turgid, with 0.3 to 0.5 mm long embryos (Fig. 1*a*). Although 30 DAP caryopses looked shrunken, embryos had grown up to 1 - 1.5 mm long.

Twenty days after plating, 100 % of the younger embryos gave rise to white compact embryogenic calli. These turned partially green under light, and coleoptiles emerged from scutellar-like bodies (Fig. 1*b*). Subsequently, leaf-like structures, shoots and roots were observed. Embryogenic or organogenic callus frequencies did not differ among hormonal treatments (Table 1), but they decreased, on average, from 90.5 to 44.3 %,

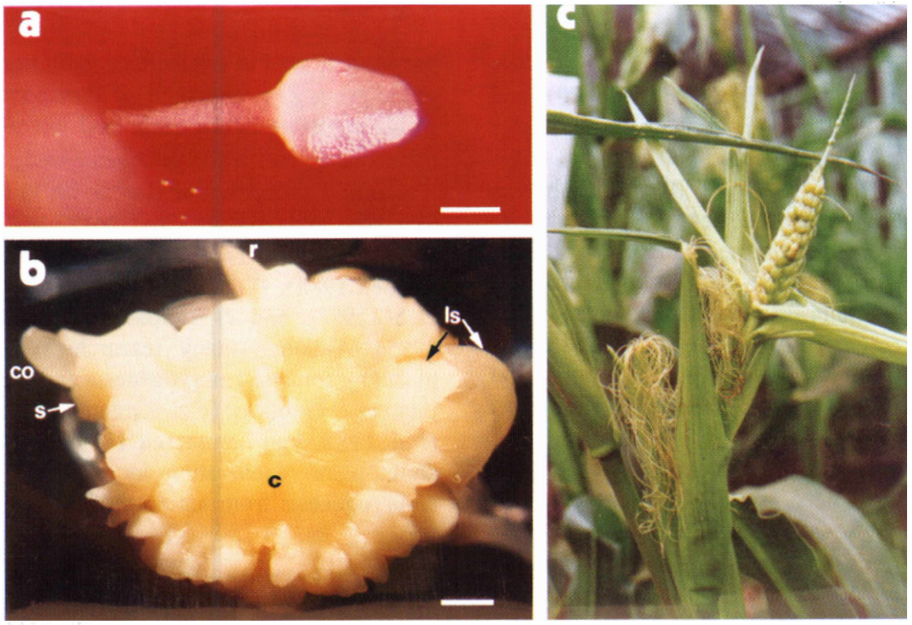

Fig. 1*a*. Isolated *Zea mays* ssp. *mays* (2n=40) × *Zea mays* ssp. *parviglumis* hybrid embryo 23 d after pollination, *bar* = 200 μm;
Fig. 1*b*. Somatic embryo (*co* - coleoptile, *s* - scutellar like body), leaf like structures (*ls*), root (*r*) and compact callus (*c*) development from the scutellum of the same embryo, 30 d after plating on 2,4-D containing medium, *bar* = 1000 μm.
Fig. 1*c*. Female inflorescences of *Zea mays* ssp. *mays* (2n=40) × *Zea mays* ssp. *parviglumis* hybrid plant.

Table 1. Frequencies of organogenic or embryogenic calli from 0.3 - 0.5 mm long embryos of *Zea mays* ssp. *mays* (2n=40) × *Zea mays* ssp. *parviglumis* hybrids, cultivated on García *et al.* (1992) basic medium supplemented with 2,4-D and kinetin [μmol dm^{-3}]: 1 - 0 (D1); 1 - 0.5 (D1C); 2 - 0 (D2); 2 - 0.5 (D2C). Observations were made 50 DAP. Somatic embryogenesis induction frequencies did not differ among hormonal treatments ($\chi^2 = 0.9215$, $P \leq 0.05$).

	D1	D1C	D2	D2C
Organogenesis or somatic embryogenesis [%]	85.71	100.00	86.66	90.00
Average of plants regenerated per competent explant	0.27	0.21	0.80	0.33
Regenerated plants [%]	23.81	21.42	73.33	30.00
Number of plated embryos	21	14	15	10

Table 2. Frequency of germination, organogenesis and other responses from 1 - 1.5 mm long embryos of *Zea mays* ssp. *mays* (2n=40) × *Zea mays* ssp. *parviglumis* hybrids, cultivated on medium supplemented with 2,4-D and kinetin (see Table 1). Observations were made 45 DAP; * - frequencies differed among hormonal treatments ($\chi^2 = 18.78$; $P \leq 0.05$), ** - frequencies differed among media with 1 or 2 mg dm^{-3} 2,4-D ($\chi^2 = 14.88$, $P \leq 0.05$).

Response [% of embryos plated]	D1	D1C	D2	D2C
Normal germination*	31.25	61.76	14.29	14.29
Organogenesis	0	0	23.81	4.76
Shoot + radicular callus	68.75	26.47	28.57	38.10
Rhizogenic callus	0	2.94	28.70	28.57
Epinastic shoot growth	0	0	4.76	14.29
Regenerated plants**	68.75	52.94	19.04	19.04
Number of plated embryos	16	34	21	21

comparing 50- and 120-d-old cultures. However, the average number of regenerants per competent explant was markedly higher on D2 medium, as well as the ratio between regenerated plants and plated embryos (Table 1). Regenerated shoots rooted on the basic medium without plant growth regulators. By this method, thirty five plants were obtained from 60 hybrid embryos.

The older embryos gave rise to organogenic callus with low frequency and only on media supplemented with 2 μmol dm^{-3} 2,4-D. The remaining embryos showed different responses (Table 2): on media with 1 μmol dm^{-3} 2,4-D most of the embryos germinated normally or developed root callus and originated 27 normal plants. Although frequency of normal germination was markedly higher on D1C medium (Table 2), the frequency of regenerated plants did not differ significantly among media supplemented with 1 mg dm^{-3} 2,4-D ($\chi^2 = 1.12$, $P \leq 0.05$), because shoots with radical callus subsequently rooted on hormone free medium and originated normal plants. These were transplanted to the greenhouse 45 d after plating and grown to maturity. On media with 2 μmol dm^{-3} 2,4-D, embryos showed more developmental alterations, such as curled shoots or rhizogenic callus. Although 8 plants were regenerated, they did not survive.

All the regenerated plants showed a somatic chromosome number of 2n=30 (Fig. 2). Such plants showed, on average, 2 m height and 8 tillers per plant.

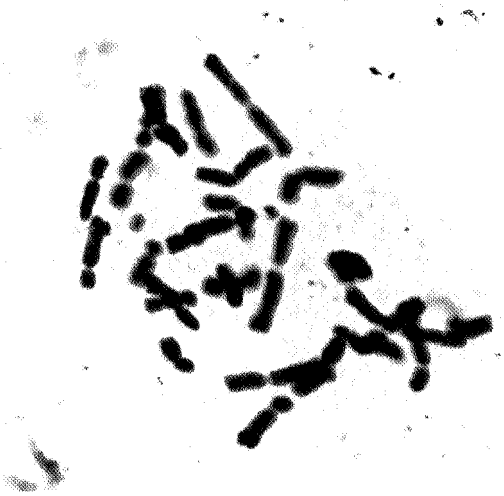

Fig. 2. Mitotic chromosomes of *Zea mays* ssp. *mays* (2n=40) × *Zea mays* ssp. *parviglumis* (2n=20) hybrid (2n=30).

They had photoperiod requirements to flower, with a short day quantitative response and a critical photoperiod of 13 h. These hybrids produce 40 to 80 % fertile pollen and 15 % viable naked caryopses. Female spikes were distichous or polistichous (4 rows), enclosed in many (6 - 8) sheaths and disposed on lateral inflorescences (Fig. 1*c*).

Discussion

Z. mays ssp. *mays* (2n=40) × *Z. mays* ssp. *parviglumis* (MPa30) hybrid embryos, obtained from crosses performed in spring, reached a 0.3 to 0.5 mm length 12 DAP (data not shown), while in autumn the same stage was reached 23 DAP. At this time caryopses looked swollen, but when embryos were 1 to 1.5 mm long (17 DAP in spring and 30 DAP in autumn), caryopses were flattened because of some endosperm development deficiencies. Randolph (1936) and Van Lammeren (1986) also observed that environmental conditions, mainly temperature, have a great effect on maize embryo growth.

Somatic embryogenesis from 0.3 to 0.5 mm MPa30 hybrid embryos (Fig. 1*b*), showed a morphology similar to that reported by Fransz and Schel (1987) for maize inbred A188 immature embryos cultured on medium supplemented with 2 mg dm^{-3} 2,4-D and 6 % sucrose. Subsequently MPa30 embryos showed proliferation of leaves, shoots and adventitious roots, characteristics of organogenesis in maize (Springer *et al.* 1979, Van Lammeren 1988). Induction frequencies of somatic embryogenesis did not differ among hormonal treatments, but the ratio between regenerated plants and plated embryos was markedly higher on D2 medium, because of the average number of regenerants per competent explant (Table 1). Plant regeneration frequencies from 0.3 to 0.5 mm hybrid embryos of different maize genotypes (2n=20 or 40) × *Tripsacum dactyloides* was also affected by the number of regenerants per competent explant and not by the somatic embryogenesis induction frequencies (García *et al.* 1999). Otherwise, the frequency of regenerated plants from the older MPa30 embryos was affected mainly by the percentage of germination and developmental alterations, which consequently affected the survival of the seedling (Table 2).

Although the induction frequency of somatic embryogenesis was almost 100 % from the younger hybrid embryos (Table 1), the older ones only regenerated plants by germination (Table 2). Most authors (*e.g.* Green and Phillips 1975, Lu *et al.* 1982, Fransz and Schel 1987, Van Lammeren 1988) described maize plant regeneration from 1 to 2 mm long embryos on media containing 5 to 10 µmol dm^{-3} 2,4-D, with different degrees of success mainly depending on the maize genotype. However, García *et al.* (1991) obtained higher frequencies of somatic embryogenesis from 0.3 to 0.5 mm long embryos, in the presence of 1 µmol dm^{-3} or less 2,4-D. The embryogenic response also decreases with the age of the explant in maize and *Z. mays* ssp. *parviglumis* hybrids. According to Erdelská and Vidovencová (1994), maize embryogenesis *in vitro* can be considered as cleavage polyembryony, where 2,4-D releases scutellar cells from the developmental integrity of plant tissue for the expression of its totipotency ("decorrelation"). As younger is the embryo, easier is the return of cells to the full totipotency stage. These could explain the high frequency of somatic embryogenesis from the MPa30 younger embryos compared with the older ones, cultivated on media with the same 2,4-D concentrations. This methodology could be used to increase somatic embryogenesis from recalcitrant *Zea* genotypes.

The hybrids, with 20 chromosomes from maize and 10 from *Z. mays* ssp. *parviglumis* were maize-like plants, except in the number of tillers per plant. According to the subgeneric classification of *Zea* (Doebley and Iltis 1980), the female inflorescences of these hybrids showed some characteristics of *Z. mays* ssp. *parviglumis* and others of the cultivated maize. Hybrid plants had female spikes disposed on lateral inflorescences as *Z. mays* ssp. *parviglumis* and naked caryopses as maize. On the contrary, maize spike is solitary and terminal on primary lateral branches of stems and tillers and *Z. mays* ssp. *parviglumis* caryopses are inside the glume-covered cupulate fruitcases. Some other characteristics of the hybrid female inflorescences, such as the number of rows per spike (2 to 4 row spike in the hybrid, distichous spike in *Z. mays* ssp. *parviglumis* and polystichous spike or "ear" in maize) and the number of sheaths enclosing the spike (6 - 8 in the hybrid, 1 in *Z. mays* ssp. *parviglumis*, and 8 - 12 in maize) were intermediate between the parents.

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