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An improved system to obtain fertile regenerants via maize protoplasts isolated from a highly embryogenic suspension culture

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Summary. Regenerants from a 30-month-old haploid and a 10-month-old diploid tissue culture were cross-pollinated to generate a synthetic genotype (HE/89) with improved competence for maintenance of totipotency in various cultured explants. The HE/89 zygotic embryos developed friable, embryogenic cultures in the commonly used MS- and N6-based media without the addition of L-proline. By optimalization and changing the culture conditions, we were able to regulate the maintenance of the earlier, more synchronous (Type II) and the later, asynchronous (Type I) in vitro embryogenesis, as well as the shift between different ontogenic stages. Within 70 days after the inoculation of immature embryos a relatively homogeneous, early-embryogenic suspension culture usable for protoplast isolation was established from the initially surface-grown cultures. Using modified solutions for protoplast isolation and culture, viable protoplasts were reproducibly obtained from which plants were regenerated via defined ontogenic steps. Despite the long in vitro history of the parental genotypes, 60–70% of the more than 500 plants derived from the HE/89 protoplasts set seeds following self- or sib-pollination.

Key words: Protoplast technology – Plant regeneration – Somatic embryogenesis – Synthetic genotype

Introduction

Efficient plant regeneration from maize protoplasts can create new possibilities for the utilization of genetic manipulation techniques, such as somatic hybridization and direct gene transfer, in maize genetics and breeding. In attempts to establish maize protoplast culture systems, suspension cultures have frequently been used as sources of protoplasts. Depending on the nature of suspension cultures the cultured protoplasts could develop into calli or microcolonies (Potrykus et al. 1979; Chourey and Zurawski 1981; Kuang et al. 1983; Ludwig et al. 1985; Imbrie-Milligan and Hodges 1986), somatic embryos (Vasil and Vasil 1987; Mórocz et al. 1986; Kamo et al. 1987), nonfertile (Rhodes et al. 1988; Cai et al. 1988) as well as fertile plants (Shilito et al. 1989; Prioli and Söndahl 1989).

Selection of fast-growing, friable embryogenic tissues can be a prerequisite for initiation of embryogenic suspension culture. As shown previously, the formation of friable embryogenic cultures was influenced by more frequent passages, low light intensity, and modification of medium composition, e.g., elevation of proline content (Green 1982, Amstrong and Green 1984); in other cases they can occur spontaneously (Lowe et al. 1985) or as a result of selection from mixed embryogenic cultures (Hodges et al. 1985). However, the embryogenic character was found to be controlled by the genotype of donor plants also (Tomes and Smith 1985; Lupotto 1986).

In this publication we report on a efficient rapid regeneration of fertile plants from maize protoplasts. We further demonstrate the potential of a breeding program in improving the tissue culture behavior of this crop. Crossing between synthetic germ plasms preselected for positive, long-term tissue culture response could result in a hybrid embryo with high embryogenic capacity in suspension culture before and after protoplast isolation.

Materials and methods

Plant material

The highly embryogenic culture HE/89 used in these studies was obtained from a hybrid embryo of less than 1 mm in size, after

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cross-pollination of the receptive silks of the regenerated H229 plants with pollens of regenerated OK281 plants. The H229 haploid, friable embryogenic culture was derived from anther cultures by S. Mórocz in 1986 (unpublished results) and maintained for 30 months before the plants were regenerated for this cross-pollination. The OK281 parental male plants were also regenerated from a friable embryogenic culture 10 months after initiation from embryo culture of an o_2o_2 synthetic genotype developed in a five-generation crossing program from the following sources: H temp Ao_2o_2, $Oh43o_2o_2$, A188, GK3/H, W64A, Mangelsdorf's Tester, and a white flint local variety.

Media and solutions

The N6M callus culture medium contained the macro elements, glycine, and vitamins of N6 medium (Chu et al. 1975), the chelated iron and micro elements of MS medium (Murashige and Skoog 1962), 500 mg/l Bacto Trypton, 3%, sucrose and 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). The pH was adjusted to 5.8 ± 0.04 by addition of ca. 40 µl 5 *N* KOH per liter; the medium was then autoclaved. The MSG medium contained the constituents of MS medium as published by Green and Phillips (1975) with 1 m*M* L-aspargine, 500 mg/l Bacto Trypton, 2 mg/l 2,4-D, and 2% sucrose. The pH was adjusted to 5.6 ± 0.04 before autoclaving. The N6M and MSG media were solidified with 0.2–0.4% gelrite or 0.8% agar for surface cultures and used in 35 ± 5 and 50 ± 5 ml quantities in 9×1.5 cm plastic and 10×2 cm glass petri dishes, respectively.

The protoplast culture medium ppN6M/89 consisted of the components of N6M medium with the following modifications (final concentrations per liter): 370 mg $MgSO_4 \cdot 7H_2O$, 300 mg $CaCl_2 \cdot 2H_2O$, 1 g sucrose, 0.4 mg 2,4-D, and additives 30 g fructose, 50 g glucose, 2.5 g maltose, 2.5 g galactose, 0.5 g galacturonic acid, 0.5 g gluconuronic acid, 500 mg L-asparagine, 100 mg L-glutamine, 100 mg L-serine, 0.7 mg naphthalene-acetic acid, and 0.7 mg zeatin-mixed isomers per liter. This medium was used without pH adjustment after filter-sterilization. As an alternative preparation method the glucose, sucrose, and fructose were autoclaved in distilled water producing 90% of the final medium, and the other constituents were combined into a tenfold concentration, filter-sterilized, and added after cooling to the autoclaved sugar solutions.

From the antioxidant n-propyl gallate (npg), a 5 mg/ml stock solution was prepared by dissolving the weighed compound with ethanol, making up 20% of the final volume before the addition of bidistilled water. After filter-sterilization this stock solution was added to the washing and isolation solutions and to the 0.6 *M* sucrose solution at 50 mg/l working concentration. The npg stock soslution was always prepared freshly, and was added to the solutions directly before use. The washing, solution (WS) contained the N6M medium without $(NH_4)_2SO_4$, iron complex, and 2,4-D and with 1% sucrose (final concentration), 5.5% glucose, and 5.5% fructose. The sugars were autoclaved and, after cooling, they were mixed with the tenfold concentration of other constituents. From bovin serum albumin (BSA, fraction V., Merck) a 40 mg/ml stock solution was prepared and kept deep frozen before use. For the preparation of desalted enzyme solution, 4 g cellulase RS and 0.2 g Pectolyase Y23 were dissolved in bidistilled water at 4 °C, the unsolved ingredients were pelleted by centrifugation, the supernatant was desalted on a 50-g swollen Sephadex G25 column with 100–300 µm bead size, the first 50-ml fraction was collected and filter-sterilized. Fifty milliliters of osmotic solution (OS) contained the following ingredients: 2.02 g KNO_3, 1.36 g KH_2PO_4, 0.47 g K_2HPO_4, 4.0 g glucose, 3.6 g fructose, and 0.34 g L-proline; it was then filter-sterilized. The protoplast isolating solution (IS) was prepared just prior to use by mixing the components in the following order: 0.3 ml desalted enzyme solution, 2.0 ml WS, 4.4 ml sterile, bidistilled water, 0.5 ml BSA, 0.1 ml npg, 0.1 ml 1 *M* $CaCl_2$, 0.1 ml 1 *M* $MgSO_4$ and 2.5 ml OS. to make 10 ml.

Establishment and maintenance of He/89 culture

Embryos less than 1 mm obtained from a greenhouse-grown plant 12 days after pollination were inoculated on N6M medium for 3 weeks, followed by two subcultures on N6M and MSG medium at 10- to 14-day intervals before a suspension culture was started in N6M medium. For regular maintenance, 4 g of cells was used to start a new suspension culture cycle of 5–7 days in 50 ml N6M medium in 100-ml Erlenmayer flasks. All cultures were grown under continuous or periodical illumination by fluorescence tubes at 22–25 °C.

Protoplast isolation and culture

Two grams of fresh-weight cell material from 2-day-old suspension culture was squashed with forceps in 1 ml WS + npg solution to eliminate larger (2–3 mm) colonies and then washed twice with 10 ml of this solution. The washed cells were incubated in 10 ml IS for 4 h in the dark with or without gentle shaking. After the enzyme treatment, the isolates were passed through a 210-µm tetex and 60-µm polymon sieve, followed by centrifugation at a standard 1,000 rpm. The pellet was resuspended in 10 ml of 0.6 *M* sucrose + npg, and 1 ml of WS + npg was overlayed for the second centrifugation. The floated protoplasts were collected and pelleted again in 15 ml WS + npg in the third centrifugation. The number of protoplasts was counted using a Buerker chamber before the third centrifugation. The protoplasts were cultured in 2 ml of liquid medium or were embedded in low-gelling-temperature agarose (Sigma) by mixing the protoplasts gently with 2 ml of a 1:1 ratio, double-strength ppN6M/89 and 1.2% melted agarose solution, after a previous temperature adjustment at 45 °C for both solutions. The protoplasts were cultured under dim light at 25 °C. For feeding protoplasts in the liquid medium, 0.5 ml N6M medium was added after 3 weeks, followed by the addition of 1 ml in 2–3 days, then after 3–4 days the whole culture was put into 10 ml fresh N6M medium and shaken. The embedded cultures were placed either on solidified N6M with or without 2,4-D or into 10–50 ml liquid N6M medium after 2–3 weeks.

Plant regeneration and growth

From the surface-grown cultures, 0.2–1 g of embryogenic callus was placed into covered glass jars, containing an aerating sponge plug, onto 100 ml of N6M lacking 2,4-D. This step was repeated until well-developed plants were obtained from the embryoids and the small plantlets. The larger plants (above 7 cm) were removed for transplantation into pots of different sizes containing various kinds of horticultural soils rich in humus. The plants with emerged silks and pollen-shedding tassels were self- or sib-pollinated.

Results

Regulation of morphogenic response in HE/89 cultures

Similar to the cultures of the parental genotypes, the inoculated HE/89 hybrid embryos formed a predominantly friable embryogenic culture. The developmental stage of the embryoids during proliferation could be regulated by the frequency of passages. In the case of subcultures at 10- to 14-day intervals, a highly homogenous,

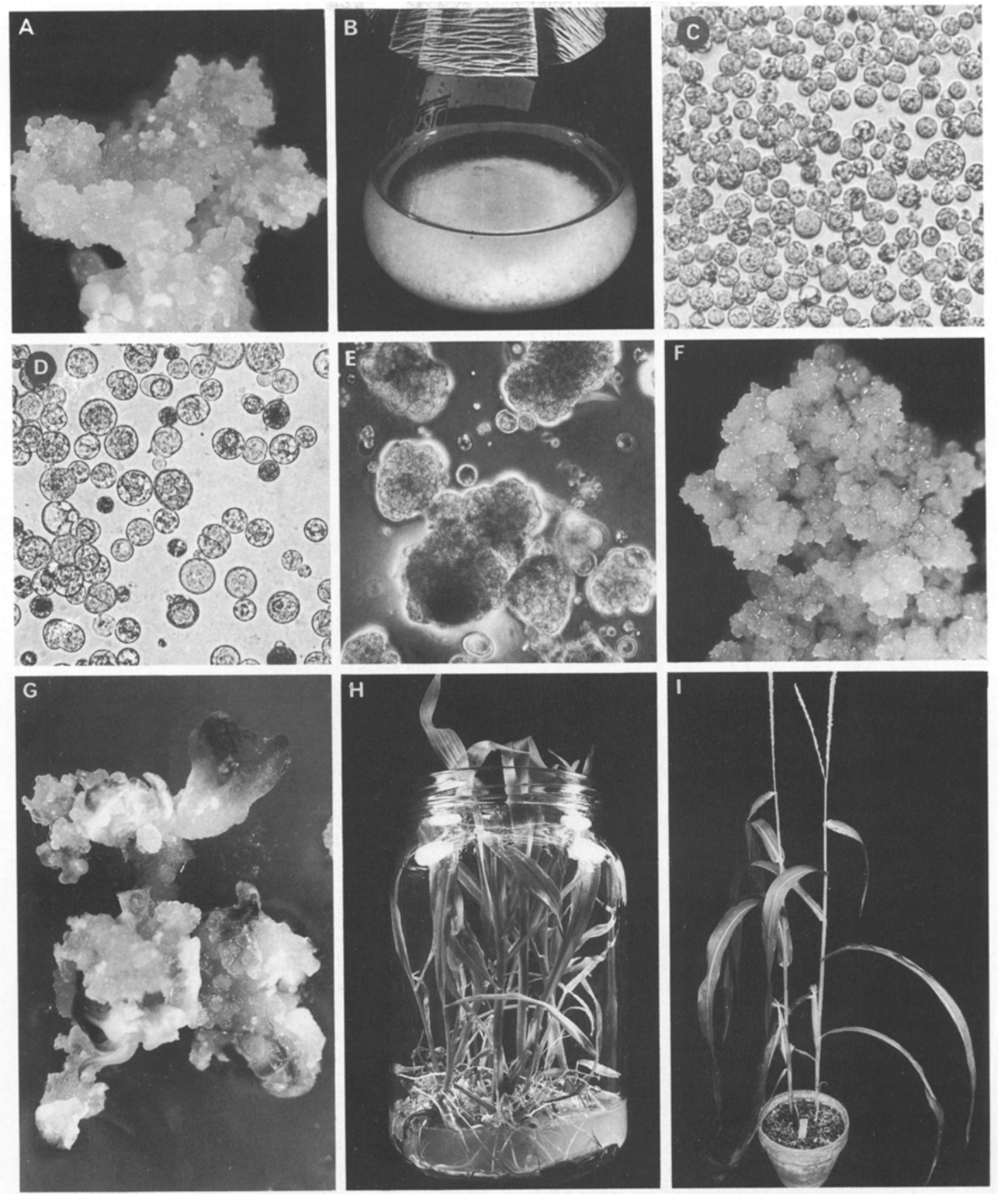

Fig. 1 A–I. The HE/89 embryogenic maize culture and the regeneration of normal plants from it via protoplast culture process. **A** Established early embryogenic culture; **B** semifine suspension culture ready for protoplast isolation; **C** protoplasts 5 h after isolation; **D** the first divisions, 32 h after isolation; **E** colonies 8 days after isolation; **F, G** early- and late-embryogenic cultures from protoplasts after 2 months, respectively, regenerated plants from protoplasts before (**H**) and after (**I**) transplantation to soil

friable culture was obtained, which represented the early developmental stage of embryoids (Fig. 1 A). When these early-embryogenic cultures were exposed to a longer incubation (e.g., 3–4 weeks) on the same medium, they developed into late-embryogenic cultures with asynchronously differentiating embryoids. The more mature of them exhibited not only greening and/or whitish, scutellar-like body formation, but also differentation of small shoots and roots. The use of MSG medium containing a higher level of 2,4-D than the N6M medium helped to shift the late-embryogenic culture to an early-embryogenic one and to keep this less developed, homogeneous embryogenic stage during maintenance. The establishment of a uniform, early-embryogenic culture on N6M medium required more frequent passages because this medium tended to promote the maturation of embryoids more than the MSG. However, once the homogeneous, early-embryogenic culture was achieved it could be easily maintained either on N6M or MSG medium with less frequent subcultures. From the late-embryogenic structures, plants were obtained after one growth cycle on 2,4-D-free N6M medium, while from the early-embryogenic structures a late-embryogenic culture was initially formed with only a few, small little regenerants; a second growth period on this medium, which lacked growth regulators, was necessary to obtain a number of well-developed plants.

Establishment of suspension culture

The growth of the established, early-embryogenic callus culture continued in the liquid shake culture without a lag period. Within 2 weeks after initiation, a semifine suspension culture with colonies 0.07–3 mm in diameter was formed that could be used for protoplast experiments (Fig. 1 B). In the first measured growth cycle at 8% (w/v) inoculation rate, the fresh weight doubled in less than 4 days. This propagation rate, with about $\pm 20\%$ variation from flask to flask, was retained in the suspension culture after 6 months of continuous maintenance under the given conditions. A similar propagation rate was observed in the suspension cultures initiated later from the surface-grown original or from protoplast-derived, early-embryogenic cultures. The fresh weight in the suspension culture reached a twofold and a tenfold times increase after less than 36 h and 8 days, respectively, when a 0.4% inoculation rate was applied.

Isolation and culture of protoplasts

The first protoplast isolation and successful culture was carried out from a 3-week-old suspension culture 70 days after the inoculation date of the donor hybrid embryo. During the enzyme treatment occasional protoplast formation was detected after half an hour, however, the majority of protoplasts was released after 2 h. At the end of a 4 h digestion period, $2-6 \times 10^6$ purified protoplasts could be obtained from the initial 2 g cells without heavy shaking or pipetting of the isolates prior to pouring through the protoplast sieves. The protoplasts were cultured either in liquid or embedded in solidified ppN6M/89 medium with a density of $0.5-1.5 \times 10^6$ protoplasts per ml and first divided cells on the 2nd day of culture, giving rise to multicellular colonies within 8 days (Fig. 1 C–E). The colony formation frequency ranged between a calculated 0.5 and 8%. The main difference between the cultivation of protoplasts in liquid or solidified medium was found in the timing of further handling of the obtained colonies. The colonies in the liquid medium required stepwise feeding and 1 week longer exposition to the initial protoplast medium before dilution or inoculation into new medium. The embedded colonies could be distributed into two or three culture vessels with liquid or solidified new media after 2 weeks, although they formed a suspension culture or a continuous lawn more readily (Fig. 1 F) if they were left in the initial medium for 3 weeks. When the protoplast-derived colonies were fed or placed into new media before the given exposition time in the original protoplast culture medium, they either needed a long (several weeks) incubation for further growth or failed to develop further.

Regeneration and growth of plants derived from protoplasts

The 3- to 4-week-old colonies developed from protoplasts started to form a homogenous, early-embryogenic callus culture. When the protoplast-derived colonies were placed onto solidified N6M 2,4-D-free medium, they differentiated into late-embryogenic morphological structures with occasional small shoots within 2 months of isolation (Fig. 1 G). When these germinating embryoids were transferred to new N6M 2,4-D-free medium, strong plants developed (Fig. 1 H). More than 90% of the regenerated plants obtained via protoplast culture survived transplantation to soil and developed into normal-looking plants (Fig. 1 I). Although some tassels failed to pollinate (less than 10%) and dwarf plants occasionally occurred (about 1%), 60–70% of the more than 500 plants derived from protoplasts set seeds following self- or sib-pollination.

Discussion

In this paper we demonstrate that redifferentiation of fertile plants via protoplast techniques in maize can be significantly improved by the use of responsive genotypes, as well as by the modification of culture techniques. As in all the earlier work, our results also emphasize the importance of the genotype. Both of the parental germ plasms (H229, OK281) used as crossing partners to

generate the highly embryogenic material HE/89 were the results of systematic selection for favorable tissue culture response. The combination of advantageous gene pools through a crossing program and the selection under culture conditions allowed us to develop materials with high embryogenic potential, long-term plant regeneration capacity, and stability of regenerated plants. The advantage of HE/89 culture in protoplast work was shown by the relatively short period of time required to develop a proper suspension and by the necessity of low enzyme concentration (Cellulase RS 0.24%, Pectolyase Y23 0.012% in w/v) to isolate protoplasts. As in the work of earlier investigators, the establishment of embryogenic suspension culture was a basic requirement to obtain protoplasts for further culture. In addition to the key role of the present genotype, here we also tried to improve the conditions for both protoplast isolation and culture. In order to decrease the harmful effect of enzyme treatment (Hanne and Lörz 1988), we used reduced concentrations of desalted enzymes. Based on the results published by Saleem and Cutler (1986), we applied n-propyl gallate to inhibit lypoprotein oxidation in cell membranes, but we used it only during the isolation process.

Frequently in tissue culture experiments the use of the nurse culture techniques or conditioned medium was found to be beneficial in obtaining colony formation with sufficient efficiency (Kuang et al. 1983; Ludwig et al. 1985; Rhodes et al. 1988; Shilito et al. 1989; Somers et al. 1987). In other cases the protoplasts developed into colonies without nurse culture techniques (Chourey and Zurawski 1981; Vasil and Vasil 1987; Mórocz et al. 1986; Kamo et al. 1987; Cai et al. 1988; Shilito et al. 1989; Prioli and Söndahl 1989). Although in the experiments presented here we did not need to use nurse culture techniques or conditioned medium, one possible element of conditioning factors was included in the ppN6m medium. Based on the observations of pH decrease shortly after subculture in different maize suspension cultures (unpublished results), we did not change the low pH caused by sugare acids in the ppN6M/89 protoplast culture medium, since we suggest that the initial low pH in the protoplast medium can help to recover from these stresses of protoplastation. Other media such as KM8p (Kao and Michayluk 1975) and different modifications of ppN6M/89 medium also led to successful protoplast culture (data not shown), but further experiments are needed to prove the advantage of protoplast medium ppN6M/89.

As far as the plant regeneration pathways in the embryogenic maize cultures such as HE/89 are concerned, we suggest that the friable embryogenic culture that was frequently referred to as type II culture also represents the early-embryogenic stage in the ontogeny of in vitro embryoids. Later maturation and further differentiation of these early-embryogenic, morphological structures lead to the formation of compact, morphologically compound cultures referred to as type I culture, which also have scutellar-like bodies, small shoot and root formation. While the early-embryogenic cultures grow homogenously without any sign of differentiation, the late-embryogenic cultures are composed from different ratios of less or more maturated and germinated, polyembryogenic morphological structures. Therefore, we preferred to discriminate between the two stages of HE/89 callus cultures as early and late embryogenic. The use of this terminology in this article was supported by the fact that these stages were interchangeable and were regulated by the frequency of passages and the composition of the culture medium.

The use of the described, highly morphogenic material HE/89 further broadens the range of maize germ plasms suitable for fertile plant regeneration from protoplasts. This genotype, together with the given isolation and culture protocol, led to successful culture in all of the more than 20 experiments carried out in three independent laboratories. The protoplast-plant system presented here can be used for different kinds of genetic manipulations, such as protoplast fusion or genetic transformation.

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References

Amstrong CL, Green CE (1984) Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-proline. Planta 164:207–214

Cai Q, Kuo C, Quian Y, Jiong R, Zhon Y (1988) Somatic embryogenesis and plant regeneration from protoplast of maize (*Zea mays* L.) In: Puite KJ, Dons JJM, Huising HJ, Kool AJ, Koornneef M, Krens FA (eds) Progress in plant protoplast research. Kluwer Academic Publishers, Dordrecht, p 120

Chourey PS, Zurawski DB (1981) Callus formation from protoplasts of a maize cell culture. Theor Appl Genet 59:341–344

Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, Chu CY, Bi FY (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci Sin 18:659–668

Green CE (1982) Somatic embryogenesis and plant regeneration from the friable callus of *Zea mays*. In: Proc 5th Int Congr Plant Tiss Cell Cult. Fujiwara, Tokyo, pp 107–108

Green CE, Phillips RL (1975) Plant regeneration from tissue cultures of maize. Crop Sci 15:417–421

Hanne G, Lörz H (1988) Release of phytotoxic factors from cell walls during protoplast isolation. Plant Physiol 132:345–350

Hodges TK, Kamo KK, Becwar MR, Schroll S (1985) Regeneration of maize. In: Zaitlin M, Day P, Hollander A (eds.) Biotechnology in plant science. Academic Press, New York, pp 15–33

Imbrie-Milligan CW, Hodges TK (1986) Microcallus formation from maize protoplasts prepared from embryogenic callus. Planta 168:395–401

Kamo KK, Chang KL, Lymm ME, Hodges TK (1987) Embryogenic callus formation from maize protoplasts. Planta 172:245–251

Kao KN, Michayluk MR (1975) Nutrient requirements for growth of *Vicia hajastana* cells and protoplasts at very low population density in liquid media. Planta 126:105–110

Kuang VD, Shamina ZB, Butenko RG (1983) Use of nurse tissue culture to obtain clones from cultured cells and protoplasts of corn. Fiziol Rast 30:803–812

Lowe K, Taylor DB, Ryan P, Paterson KE (1985) Plant regeneration via organogenesis and embryogenesis in the maize inbred line B73. Plant Sci 41:125–132

Ludwig SR, Somers DA, Petersen WL, Pohlman RF, Zarowits MA, Gengenbach BG, Messing J (1985) High frequency callus formation from maize protoplasts. Theor Appl Genet 71:344–350

Lupotto E (1986) In vitro culture of isolated somatic embryos of maize (*Zea mays* L.). Madica 31:193–201

Mórocz S, Dudits D, Németh J (1986) Two approaches to rendering *Zea mays* L. applicable to tissue culture manipulations. In: Somers DA, Gegenbach BG, Biesboer DD, Hackett WP, Green CE (eds) VI. International Congress on Plant Tissue and Cell Culture, Minneapolis MN, University of Minneosta, p 190

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Potrykus I, Harms CT, Lörz H (1979) Callus formation from cell culture protoplasts of corn (*Zea mays*, L.). Theor Appl Genet 54:209–214

Prioli LM, Söndahl MR (1989) Plant regeneration and recovery of fertile plants from protoplasts of maize (*Zea mays* L.). Bio/Technol 7:589–594

Rhodes CA, Lowe KS, Ruby KL (1988) Plant regeneration from protoplasts isolated from embryogenic maize cell cultures. Bio/Technol 6:56–60

Saleem M, Cutler AS (1986) Stabilizing corn leaf protoplasts with n-propyl gullate. J Plant Physiol 128:479–484

Shilito RD, Carswell GK, Johnson CM, DiMaio JJ, Harms CT (1989) Regeneration of fertile plants from protoplasts of elite inbred maize. Bio/Technol 7:581–587

Somers DA, Birnberg PR, Petersen WL, Brenner ML (1987) The effect of conditioned medium on colony formation from "Black Mexican Sweet" corn Protoplasts. Plant Sci 53:249–256

Tomes DT, Smith OS (1985) The effect of parental genotype on initiation of embryogenic callus from elite maize (*Zea mays* L.) germ-plasm. Theor Appl Genet 70:505–509

Vasil V, Vasil IK (1987) Formation of callus and somatic embryos from protoplasts of a commercial hybrid of maize (*Zea mays* L.). Theor Appl Genet 73:793–798