Theor Appl Genet (1989) 77:820–824

Artificial seeds in barley: encapsulation of microspore-derived embryos

S. K. Datta and I. Potrykus

Institute for Plant Sciences, Swiss Federal Institute of Technology, ETH-Zurich, CH-8092 Zurich, Switzerland

Received November 14, 1988; Accepted January 9, 1989
Communicated by G. Wenzel

Summary. An in vitro culture system has been developed for barley (*Hordeum vulgare*), which yields high frequencies of high quality microspore-derived embryos without an intervening callus phase. The embryos are very similar to zygotic embryos with regard to their morphology and germination capacity. These embryos were encapsulated in sodium alginate to produce individual beads containing one embryo each. In accordance with the literature, these beads are denoted "artificial seeds". The artificial seeds germinated well and with a root system superior to that of non-encapsulated embryos. The artificial seeds also maintained their germination capacity for at least 6 months, whereas non-encapsulated embryos did not survive more than 2 weeks in storage. Artificial seeds, thus, probably provide a simple and universal delivery system of in vitro plantlets to greenhouse or field.

Key words: Artificial seeds – Direct microspore embryogenesis – Storage – *Hordeum vulgare* – Haploidy

Introduction

Haploid plant breeding is well established as a tool for crop improvement (Wenzel and Foroughi-Wehr 1984; Dunwell 1986; Morrison and Evans 1988). Its general application is, however, still limited because: (1) not every plant species or variety responds readily to the inductive conditions, (b) regeneration frequency is often poor, (c) it leads to albino plantlets, and (d) plants regenerated are often abnormal. Our recent success in the regeneration of large numbers of green and fertile plants from cereal microspores via direct embryogenesis (Datta and Potrykus 1988), and that of others in barley (Hunter 1987; Olsen 1987), may contribute to a wider application of haploid plant breeding; it also allowed us to approach the formation of "artificial seeds" in cereals. Artificial seeds, consisting of culture-derived embryos encased in a protective coating are considered to allow for the economical mass production of elite plant varieties (Fuji et al. 1987). For dicots there are several reports which support this consideration (Kitto and Janick 1985; Redenbaugh et al. 1986; Fuji et al. 1987; Nouaille and Petiard 1988). There is also a recent report on the regeneration of sandalwood from artificial seeds (Bapat and Rao 1988). However, there is to our knowledge no report on the production of artificial seeds in cereals. In the following communication, we report on the mass development of high quality single microspore-derived embryos and their use in the production and germination of artificial seeds in barley.

Materials and methods

Hordeum vulgare var. IGRI was used throughout the study. Spikes were harvested from plants grown in our greenhouse, or were sent to us from F. L. Olsen, Carlsberg Laboratories, Copenhagen, harvested from plants grown under optimal conditions (Olsen 1987). Spikes were maintained for 4 weeks at 4 °C in the dark (Huang and Sunderland 1982) prior to the isolation and culture of the anthers at the uninucleate stage (Xu and Sunderland 1981). Anthers were floated on FW culture medium (Foroughi-Wehr et al. 1976), based on MS medium (Murashige and Skoog 1962), and further modified with 20% w/v Ficoll 400, 750 mg/l glutamine, 0.2 mg/l indoleacetic acid, 1 mg/l thiamine-HCl and incubated at 25 °C in continuous dark. It was also important either to pool 30 anthers per 10 ml of culture medium in 50 mm STERILIN plastic dishes (Olsen 1987) or 15 anthers per 1 ml of culture medium (Datta and Wenzel 1988). After about 5–6 weeks of incubation, microspore-derived proembryos and embryos at different developmental stages floated freely on the culture medium. Complete embryos with scutellum and coleoptile were taken either for germination studies or for

encapsulation. For germination, embryos were transferred to FM medium modified as above, however, without hormones and Ficoll, and with 0.5% (w/v) activated charcoal and 0.8% (w/v) Seaplaque agarose. For encapsulation, embryos were taken up in sodium alginate of different commercial sources and at a range of concentrations (data not given), dissolved in water by autoclaving, and dropped individually into a solution of 50 m*M* $CaCl_2$ in water (autoclaved) (Redenbaugh et al. 1986). Calcium-alginate beads formed around the embryos within 15–20 min at 25 °C on a rotatory shaker at 60 rpm. The optimum concentration of sodium alginate was chosen to produce capsules of suitable hardness and this varies with different commercial sources (Table 1). Thereafter, the alginate-embedded embryos (artificial seeds) were rinsed in water, dried with sterile filter paper and were either allowed to germinate directly or stored. Germination was on modified FW medium (as described above). Storage was at 4 °C in the dark in STERILIN petri dishes as used for anther culture and sealed with NESCOFILM. Seedlings were obtained from both non-encapsulated embryos and artificial seeds, vernalized for 8 weeks at 4 °C in cool fluorescence light of about 2,000 lux, 16/8 h photoperiod and, thereafter, transferred to the greenhouse. The regenerated plants were first grown in a growth chamber at 16 °C for 14 h at day and 12 °C for 10 h at night, before being transferred to the greenhouse at 20° and 16 °C during day and night with 16/8 h photoperiod, respectively. The light intensity was adjusted to 10,000 lux with Phillips HPL, 450 W bulbs.

Table 1. Comparison of various sources and concentration of sodium alginate used for producing capsules of suitable hardness*

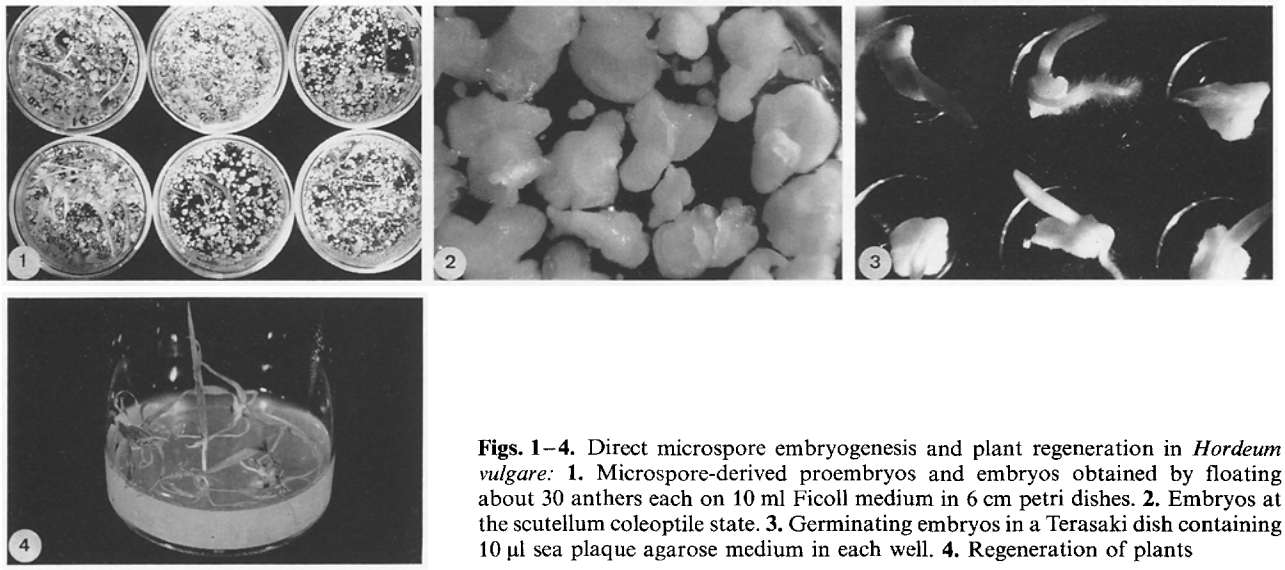
Source	Suitable conc. (% w/v)	$CaCl_2$ conc.
BDH	3.2	50 m*M*
Sigma	3.4	50 m*M*
Fluka	6.8	50 m*M*

* Only optimum concentration of sodium alginate (medium viscosity) are mentioned here

Results

Anthers floating on the liquid culture medium released microspores, microspore-derived proembryos and embryos into the culture medium where they continued to develop further (Fig. 1). In the cases where the release was very early, the development from the first division through the different stages of maturation into single bipolar embryos with scutellum and coleoptile could be followed with individual units in the microscope. In numerous cases observed in their development, there was no deviation from a direct development of one bipolar embryo from one microspore. Callus formation or development of several embryos from one unit was not observed. However, a fraction of about 1/5 of the proembryos did not complete the development to mature embryo and was finally discarded. Preculture of plants in the greenhouse as compared to growth in a controlled growth chamber at optimized conditions (Olsen 1987) did not affect the quality of development; it reduced, however, the total number of embryos and plants recovered from a given number of anthers. Greenhouse material yielded only about 20% of the embryos as compared to growth chamber material. From about 3,000 anthers plated, 660 responded with the release of developing microspores. Of those, 3,680 embryos were harvested and subjected to germination conditions. These yielded 860 green plants with roots. Sixty-six of these plants were transferred to the greenhouse and 53 of them (about 80%) were fully fertile.

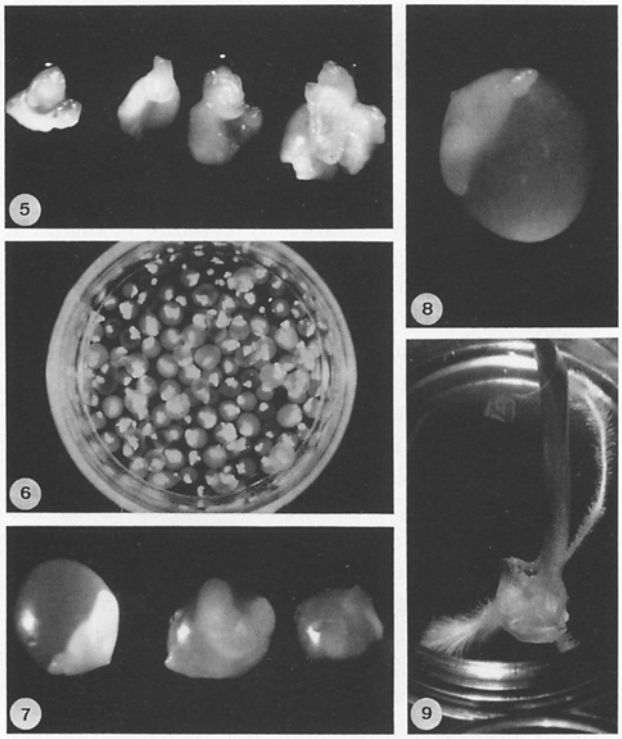
Embryo maturation often suppressed the development of proembryos in mixed populations containing different developmental stages if maintained together for a longer time. However, if the germinating embryos were separated from the proembryos and the culture was re-



Figs. 1–4. Direct microspore embryogenesis and plant regeneration in *Hordeum vulgare:* **1.** Microspore-derived proembryos and embryos obtained by floating about 30 anthers each on 10 ml Ficoll medium in 6 cm petri dishes. **2.** Embryos at the scutellum coleoptile state. **3.** Germinating embryos in a Terasaki dish containing 10 µl sea plaque agarose medium in each well. **4.** Regeneration of plants

plenished by replacing ca. 50% of the old medium with an equal volume of fresh culture medium, the retarded proembryos continued to develop and to mature.

Mature embryos with scutellum and coleoptile (Fig. 2) germinated well when transferred to the FW culture medium ('Material and methods') (Figs. 3 and 4).



Figs. 5–9. Artificial seed formation and conversion to plants in (*Hordeum vulgare*). **5.** A few selected microspore derived embryos before encapsulation with the reaction mixture of sodium alginate and $CaCl_2$ solution. **6.** Embryos encapsulated in alginate beads, each capsule contains one embryo. **7.** A few artificial seeds showing details of the embryos. **8.** The shoot emerges through the alginate matrix under germination condition. **9.** Germinated artificial seed

Alternatively, they could be used for the production of artificial seeds (Figs. 5 and 6).

Table 2 gives a comparison of the conversion frequency of encapsulated versus non-encapsulated embryos using 1,396 embryos, which produced 624 plants. The embryos were derived from anthers of donor plants raised in growth chambers.

In cases where the embryos were embedded into artificial seeds (Figs. 6–9), the germination response was found to be more vigorous than that of non-encapsulated embryos. Out of 80 artificial seeds tested for germination, 64 germinated with a well-developed root system (ca. 80%) (Fig. 9 and Table 2). Twenty of these plants were potted into soil and transferred to the greenhouse, where 16 set seeds (about 80%) as spontaneous dihaploids.

Mixed populations of embryos and proembryos were stored for different periods of time at 4 °C in the routine medium for embryo development (see 'Material and methods'). Attempts to allow for germination following transfer to normal culture conditions (24 °C) already led to browning and death of the cultures after 2 weeks of cold storage. However, embryos stored as artificial seeds under otherwise identical conditions germinated well and at a frequency of 37.5% after, so far, 6 months of cold storage.

Discussion

The term "direct microspore embryogenesis" describes the phenomenon whereby, as far as can be judged from microscopic observation, individual (and competent) microspores develop into single bipolar embryos with the characteristic scutellum and coleoptile organs. It is indeed surprising how similar these embryos are compared to zygotic embryos developing from a fertilized egg cell (the zygote) within the specific and protected environment of the maturing embryo sac, including the developing endosperm. This similarity in development of an iso-

Table 2. Comparison of conversion frequency of encapsulated and non-encapsulated embryos to plants

Materials	Embryo germination to plants					
	Non-encapsulated			Encapsulated		
	Embryo cul	Embryo germ	% of germ	Embryo cul	Embryo germ	% of germ
Fresh culture (selected)*	460	285	62	80	64	80
Fresh culture (non-selected)**	680	176	26	120	78	65
6-month-old culture	–	–	–	56	21	37,5

* Selected: embryos with scutellum and coleoptile
** Non-selected: embryos randomly taken

lated single microspore floating in an unspecific liquid environment, and the zygote being embedded in the developing embryo sac indicates that the entire mechanism of regulation of embryogeny is an autonomous and self-regulated process of a single cell, which is independent from any specific regulatory mechanism in the surrounding tissue or environment.

Direct embryogenesis from cereal microspores through anther culture has been described recently for wheat (Datta and Wenzel 1988), and was further improved in terms of yield of green plants (Olsen 1987). Even culture of isolated microspores leading to plant regeneration has been reported for barley (Köhler and Wenzel 1985; Wei et al. 1986). Although this method is not yet as efficient as anther float culture, it offers advantages in cases where the experimental goal requires additional experimental treatments of early proembryos. For the purpose of production of large numbers of microspore-derived embryos, however, anther float culture is, to date, the method of choice.

Conditions important for the development of embryos from microspores are: (a) growth of the donor plants in relatively cool temperatures, (b) cold pretreatment of the spikes (Huang and Sunderland 1982; Datta 1987), (c) a culture medium with relatively low ammonium and relatively high glutamine as a source for reduced nitrogen, (d) Ficoll to keep anthers floating at the surface of the culture medium (Kao 1981; Datta and Potrykus 1988), (e) relatively low auxin to prevent development of unorganized cultures (Datta and Potrykus 1988).

Plants regenerated from unorganized cell cultures often show unwanted aberrant phenotypes including sterility, albinoism and genome mutations. It is assumed that these aberrations are favoured by prolonged culture phases at an unorganized state (callus), especially in the presence of high levels of 2,4-D. It can be assumed then that the process of direct embryogenesis might avoid these problems, and the regeneration of fertile green plants at high frequencies as reported in this study supports this assumption.

An experimental system which allows for the development of cereal embryos from single cells in a freely accessible liquid environment might also provide novel experimental opportunities for studies in the regulation of cereal embryogeny. We would like to emphasize that the system presented here provides a different quality compared to the numerous "embryogenic suspensions" described for cereals (Lörz et al. 1988; Vasil 1988), where differentiation is suppressed by high 2,4-D levels and where, upon release of this suppression, embryos develop at multiple sites from unpredicted cells out of multicellular structures.

The high frequency of green plants recovered will be of interest for the application of the technique in plant breeding programs. The conditions mentioned earlier with regard to development of embryos from microspores and the embryogenic pathway to plants may have a strong influence on the formation of normal green plants. It is not possible yet to judge whether this increased frequency of regeneration of fertile plants will contribute to a better recovery of the genetic variation produced during meiosis.

Finally, the possibility of efficiently recovering embryos and plants from single cells and few-celled proembryos in cereals laid the basis for an approach to the recovery of transgenic chimeras in cereals following microinjection of marker genes into the cells of proembryos, analogous to the experiments with *Brassica napus* where transgenic chimeras were recovered following microinjection of a marker gene into microspore-derived proembryos (Neuhaus et al. 1987). Experiments on these lines are in progress.

The microspore-derived embryos have also been used to test for cereals the concept of "artificial seeds", as only recently developed with alfalfa and carrot (Kitto and Janick 1985; Redenbaugh et al. 1986; Nouaille and Petiard 1988), on the basis of embryogenic suspensions from diploid somatic cells. It also turned out that microspore-derived embryos of cereals can be used for the production of "artificial seeds". We do not pretend that this is the beginning of mass production and propagation of elite genotypes and of vegetative propagation of individual elite cereal plants. There are, however, a few experimental advantages connected with the possibility of embedding high quality somatic cereal embryos into alginate beads. Embryos placed individually into the alginate beads develop a better root system and germinate better and can be safer grown to maturity, thus reducing the loss of possible regenerants. Artificial seeds also facilitate the handling and delivery of the embryos, and they may even be advantageous for shipment between laboratories. The most important advantage however, is, probably related to the fact that embryos in alginate beads can be stored for, to date, 6 months and possibly longer. This allows the pooling of embryos from different experiments, which in turn will allow the optimization of further handling, and – important for laboratories located at suboptimal geographic locations and not equipped with high quality greenhouses – it also allows the preservation of valuable experimental material until the proper season will allow for an optimal development of the cereal seedlings in the greenhouse or field.

Acknowledgements. We thank F. L. Olsen, Copenhagen, for providing plant materials. The study leave granted to S.K.D. from Visva-Bharati University, Santiniketan and an ETH-Fellowship are also gratefully acknowledged. We thank Dr. M. Saul, Zurich, for the correction and critical reading of the manuscript and Dr. J. Dunwell, UK, for valuable comments.

References

Bapat VA, Rao PS (1988) Sandalwood plantlets from "Synthetic seeds". Plant Cell Rep 7:434–436

Datta SK (1987) Plant regeneration by pollen embryogenesis from cultured whole spikes of barley (*Hordeum vulgare*). Theor Appl Genet 74:121–124

Datta SK, Potrykus I (1988) Direct pollen embryogenesis in cereals. Experientia (Suppl) 44:43

Datta SK, Wenzel G (1987) Isolated microspore derived plant formation via embryogenesis in *Triticum aestivum* L. Plant Sci 48:49–54

Datta SK, Wenzel G (1988) Single microspore derived embryogenesis and plant formation in barley (*Hordeum vulgare*). Arch Züchtungsforsch 18:125–131

Dunwell JM (1986) Pollen, ovule and embryo culture as tools in plant breeding. In: Withers LA, Alderson PG (eds) Plant tissue culture and its agricultural applications. Butterworths, London, pp 375–404

Foroughi-Wehr B, Mix G, Gaul H, Wilson HM (1976) Plant production from cultured anthers of *Hordeum vulgare* L. Z Pflanzenzücht 77:198–204

Fuji JA, Slade DT, Redenbaugh K, Walker KA (1987) Artificial seeds for plant propagation. TIBTECH 5:335–339

Huang B, Sunderland N (1982) Temperature stress pretreatments in barley anther culture. Ann Bot 49:77–88

Hunter KRI (1987) Plant generation method, European Patent Application No. 87200773

Kao KN (1981) Plant formation from barley anther cultures with Ficoll media. Z Pflanzenphysiol 103:437–443

Kitto S, Janick J (1985) Production of synthetic seeds by encapsulating asexual embryos of carrots. J Am Soc Hortic Sci 110:277–282

Köhler F, Wenzel G (1985) Regeneration of isolated barley microspores in conditioned media and trials to characterise the responsible factor. J Plant Physiol 212:181–191

Lörz H, Göbel E, Brown P (1988) Advances in tissue culture and progress towards genetic transformation of cereals. Plant Breed 100:1–25

Morrison RA, Evans DA (1988) Haploid plants from tissue culture: New plant varieties in shortened time frame. Bio/Technology 6:684–689

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

Neuhaus G, Spangenberg G, Mittelsten-Scheid O, Schweiger HG (1987) Transgenic rapeseed plants obtained by the microinjection of DNA into microspore derived embryoids. Theor Appl Genet 74:30–36

Nouaille C, Petiard V (1988) Semences Artificielles: Rêves et Réalités. Biofutur 67:33–38

Olsen FL (1987) Induction of microspore embryogenesis in cultured anthers of *Hordeum vulgare*. The effects of ammonium nitrate, glutamine and asparagine as nitrogen source. Carlsberg Res Commun 52:393–404

Redenbaugh K, Grian DP, Nichol JW, Kossler ME, Viss PR, Walker KA (1986) Somatic seeds: Encapsulation of asexual plant embryos. Bio/Technology 4:643–645

Vasil IK (1988) Progress in the regeneration and genetic manipulation of cereal crops. Bio/Technology 6:397–402

Wei ZM, Kyo M, Harada H (1986) Callus formation and plant regeneration through direct culture of isolated pollen of *Hordeum vulgare* cv. Sabarlis. Theor Appl Genet 72:252–255

Wenzel G, Foroughi-Wehr B (1984) Anther culture of cereal and grasses. In: Vasil IK (ed) Cell culture and somatic cell genetics of plants, vol 1. Academic Press, New York, pp 311–327

Xu ZH, Sunderland N (1981) Glutamine, inositol and conditioning factor in the production of barley pollen callus in vitro. Plant Sci Lett 23:161–168