Application of Wheat (*Triticum aestivum* L.) Microspore Culture and Ovaries to Raise Wheat Zygotes In Vitro

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Zygotes fertilized in planta developed into fertile plants in vitro. Microspore cultures and ovaries derived from the same species were tested as nurse cells. With both types of feeder system about 20% of the isolated zygotes were able to regenerate into plants. The morphology, cytological properties and development rhythm of the zygotes resembled those of the in vivo course of zygotic development, except that the first division appeared symmetrical in contrast to the asymmetrical division observable in planta. The results indicate that ovaries may have the same nurse effect as microspores on zygotes cultured in vitro. Using ovaries as a nurse system is much less time-consuming than using isolated microspore cultures.

Key words: Embryo regeneration, growth factors, nurse cells, isolated zygotes.

INTRODUCTION

Female gametes could be a suitable source for obtaining transgenic cereal plants because they are non-chimeric and it is probably easier to integrate alien DNA than with other plant material since they are naturally receptive to the DNA of the sperm cell, which is also sourced externally. To do this, egg cells must first be microinjected with a transgene (Pónya et al., 1999) and then fertilized in vitro (Kranz and Lörz, 1993). After that the transgenic zygotes need to be cultured in vitro (Kranz and Kumlehn, 1999). Although all these steps were more or less solved individually in cereals, the whole procedure has not been performed yet. Wheat gametes fertilized by electrofusion have developed into multicellular structures (Kovács et al., 1995), but growing the zygotes into plants in vitro is not yet routine practice.

In cereals, fertile plants can be raised from isolated zygotes via ovule culture (Holm et al., 1995; Kumlehn et al., 1997) or via nurse cell culture. As feeder cells, non-morphogenic cell suspensions originating from immature embryos of maize or androgenetically induced isolated microspore suspensions from barley, maize (for review: Kumlehn et al., 1998) and wheat (Bakos et al., 2002) have been applied so far. Since ovaries are used in isolated wheat microspore cultures to promote androgenic development by releasing growth factors which are as yet undetermined (Puolimatka et al., 1996), these ovaries may also serve as feeder cells for zygotes without microspores. Successful application of ovaries as nurse cells would provide a less labor-intensive culture method.

In the present study, this time-saving method was tested for in vitro cultivation of isolated wheat zygotes fertilized in planta.

MATERIALS AND METHODS

The wheat genotype Mv Plma was used for the nurse cultures (microspore culture and ovaries), and the zygotes were isolated from wheat cv. Siete Cerros.

The isolated microspore culture was carried out as described previously (Bakos et al., 2002). The method is based on the work of Olsen (1991), Puolimatka et al. (1996) and Hu and Kasha (1997).

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Florets of Siete Cerros plants were emasculated at 4 to 7 days before anthesis and hand-pollinated 0–3 days after the age at which anthesis normally takes place. Zygotes were isolated mechanically as described previously for egg cells (Kovács et al., 1994) 6 to 18 h after pollination, then embedded in agarose droplets in microtiter plates. Microspore or ovary cultures, both containing immature unpollinated ovaries, were precultured for 7 days and then layered on the agarose droplets containing the zygotes. The cultures were incubated at 26°C under 16 h dim light (50 μmol m^{-2} sec^{-1}) and were replenished weekly with 0.1 ml of medium and water mixture (1:1) to avoid desiccation. After 2 weeks the unpollinated ovaries were replaced by ovaries fertilized at the same time as the cultured zygotes. After 3 to 6 weeks of culturing, embryo-like structures originating from both zygotes and microspores were separately transferred to agarose-solidified MS medium (Murashige and Skoog, 1962). The regenerants were vernalized at 4°C for 6 weeks and then potted up and grown in climate chambers until maturation.

The viability of the zygotes was determined based on membrane integrity and cytoplasm density viewed using phase contrast in an M35 (Zeiss, Germany) inverted microscope.

RESULTS

MMS3 medium containing 90 g/l maltose proved optimal for the development of both zygotes and nurse cells, so this medium was applied for co-cultivation (Bakos et al., 2002).

The number of zygotes that can be isolated successfully is very limited, and zygotes subjected to a viability test cannot be cultured further, so viability was determined from their visible cytological structures. Viable zygotes have less dense cytoplasm and continuous membranes, while dead ones have dense, aggregated cytoplasm and injured membranes. The difference between the results obtained with this method and with the FDA probe (Widholm, 1972) was minimal (data not shown).

The viability (Fig. 1) and development (Fig. 2) of zygotes fertilized in planta were followed in co-cultures containing either microspores with ovaries (Fig. 3a), only ovaries (Fig. 3b) or only microspores (Fig. 3c). The numbers of co-cultured zygotes were 76, 50 and 57, respectively. Of the cultivated zygotes, approximately 30% were still alive after the first day in all types of co-cultures. Zygotes co-cultivated with only microspores developed into micro-
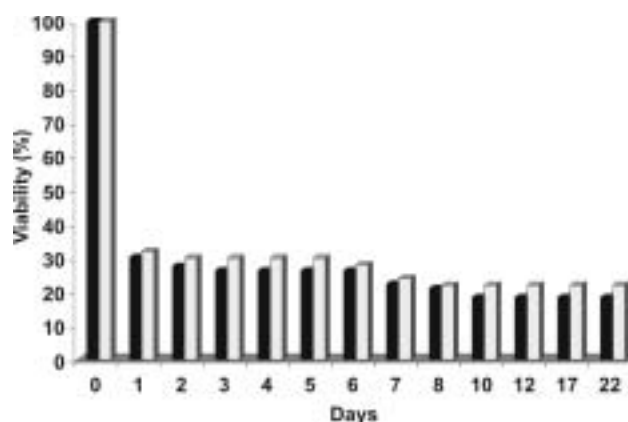

Fig. 1. Percentage of viable zygote-derived structures as a function of time. Zygotes were co-cultivated with microspores and ovaries (black columns) or only ovaries (grey columns), all of them originating from wheat.

calli and died within a week (Fig. 3d). In the cultures containing ovaries, almost all the surviving zygotes regenerated into fertile plants (Figs. 1, 4).

Irrespective of the presence of microspores, the developmental rhythms and patterns of the zygotes were very similar when the cultures contained ovaries (Figs. 1, 2). The first division of the zygotes took place after about one day of culture, and appeared symmetrical (Fig. 2b). The zygotes developed into compact structures containing small cells with dense cytoplasm by the 5th day (Fig. 2c). Club-shaped embryos (Fig. 2d) were formed by the 10th day, and dorsoventrally differentiated mature embryos (Fig. 2e) by the 18th day. All the embryos regenerated into fertile plants (Fig. 4).

DISCUSSION

The initial mortality of the zygotes may be due to osmotic shock occuring during transfer from the mannitol solution to the MMS3 medium. The work is now aimed at eliminating this initial mortality. Maës et al. (1999) examined the sugar, amino acid and ion content of the ovule and pericarp together during the first few days of in vivo embryogenesis, and their results could provide useful guidelines.

In planta, maternal gene products influence the polarity and initial divisions of the zygote, probably via genetic imprinting (Raghavan, 2000; 2001). Embryogenesis in vivo starts with asymmetrical division of the zygote, but when it was removed from the maternal tissue this first division appeared symme-
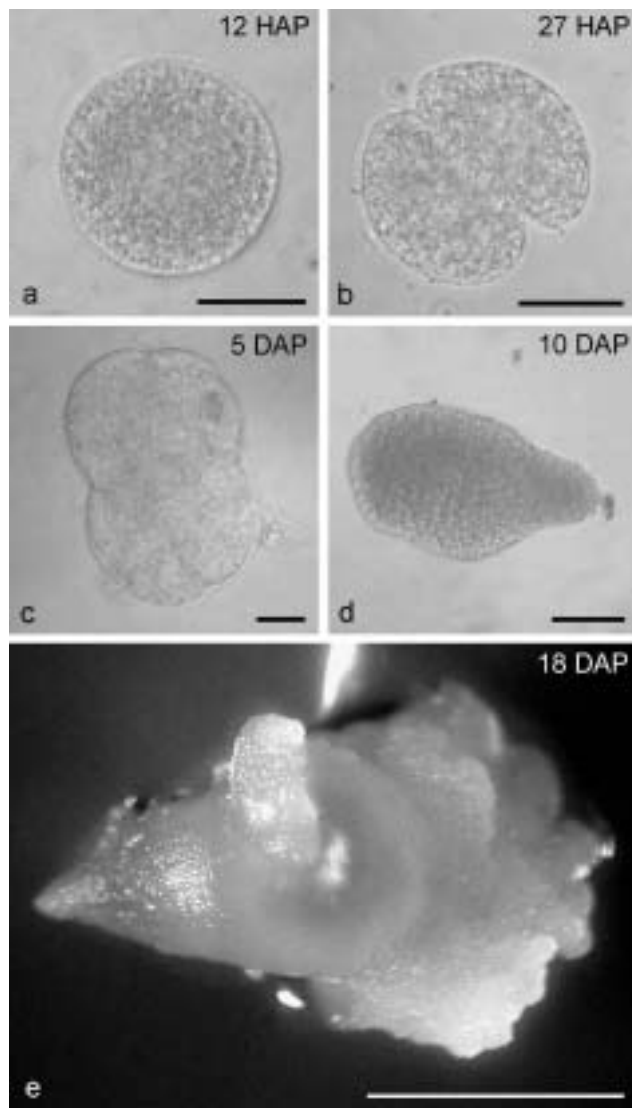
Fig. 2. The development of isolated zygotes when ovaries were used as nurse cells. (**a**) Freshly isolated zygote fertilized in planta, (**b**) Symmetrically divided zygote, (**c**) Symmetrical structure containing several small cells with dense cytoplasm, (**d**) Club-shaped embryo, (**e**) Dorsoventrally differentiated mature embryo. HAP – hours after pollination; DAP – days after pollination. Bars = 25 μm in (a–c), 100 μm in (d), 1 mm in (e).

Fig. 3. Different types of explants tested as feeder systems on cultured zygotes. (**a**) Isolated wheat microspore culture containing ovaries 14 days after microspore isolation, (**b**) Whole culture in which zygotes were co-cultivated with fertilized pistils 18 days after zygote isolation. One zygotic embryo is germinating (arrow), (**c**) Isolated microspores cultured without ovaries 8 days after microspore isolation. Microspores developed only into multicellular structures, then perished, (**d**) Microcallus developed from freshly isolated zygote in 7 days. At this stage, development stopped when only microspores were used as nurse cells without ovaries. Bars = 100 μm in (a) and (c), 2 mm in (b), 50 μm in (d).

trical (Fig. 2b), as also found by Kumlehn et al. (1999). Further divisions resulted in mostly symmetrical structures (Fig. 2c), suggesting an initial lack of polarization. However, the daughter cells were small and contained dense cytoplasm, presumably indicating initiation of embryogenic development. Although the initial starting period of in vitro development in isolated zygotes differed from zygotic development in planta, the zygotic structures later returned to the embryogenic developmental pattern (Fig. 2d,e).
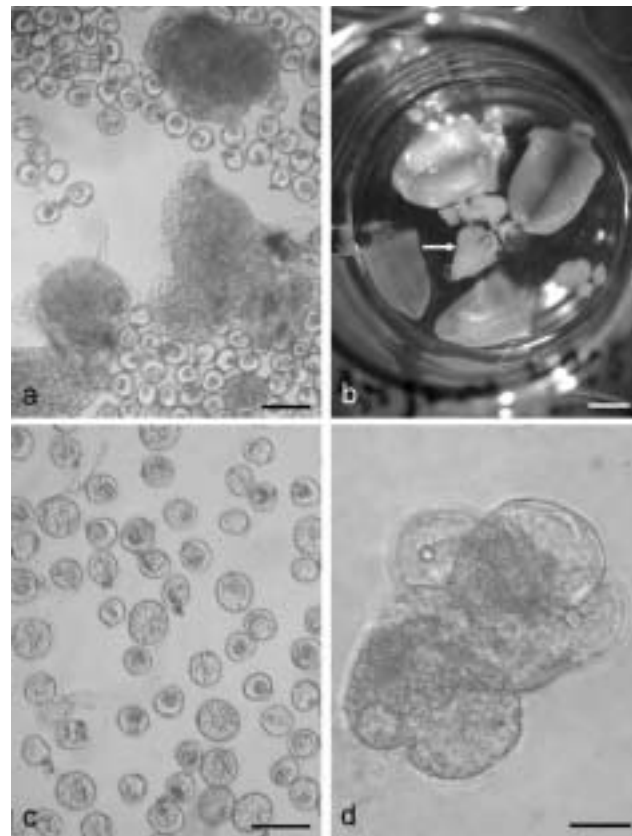
Neither viability throughout the culturing period nor the developmental pattern of the zygotes differed significantly between the two types of co-cultures containing ovaries (Figs. 1, 2). However, microspore cultures that did not contain ovaries were unsuitable as a feeder system; the zygotes did not develop much further than in a defined medium (Bakos et al., 2002). In these cultures, the development of both the microspores (Fig. 3c) and the zygotes (Fig. 3d) was restricted to only a few division cycles. This accords with the experience with microspore culture of Puolimatka et al. (1996). Several types of explants can be applied successfully as feeder systems for microspores (Puolimatka and Pauk, 1999) and zygotes (Kumlehn et al., 1998;

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Fig. 4. Flowering fertile plants regenerated from isolated zygotes fertilized in vivo. Wheat ovaries were applied as feeder system.

Bakos et al., 2002), but their density must be high enough to provide sufficient amounts of growth factors for normal development of zygotes. The quality of the excreted hormone-like compounds may also be important, since synchronicity between the developmental stages of the ovaries used as nurse cells and the cultured zygotes is necessary to the embryonic development of the isolated zygotes. Using ovaries as a feeder system requires much less work than using isolated microspore cultures or other cell suspensions. This type of nurse culture may be extended to more genotypes than is the case with microspores.