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EVIDENCE FOR MICROSPORE EMBRYOGENESIS IN WHEAT ANTHER CULTURE

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SUMMARY

In wheat, plants may be regenerated from microspores via direct embryogenesis or organogenesis or embryogenesis from callus. Light and scanning electron microscopy were used to carefully study morphogenesis of microspore-derived plants from anther culture on modified 85D12 starch medium and to determine whether the plants were formed via organogenesis or embryogenesis. Our results indicate that plants are formed via embryogenesis from microspores. Evidence for embryogenesis included the formation of the epidermis and a suspensorlike structure (21 days after culture), followed by initiation of an apical meristem, differentiation of the scutellum, and embryo elongation. At 28 days in culture, the embryo possessed a well-developed scutellum and axis with suspensor. Embryogenesis was further confirmed by coleoptile and radicle elongation during germination when the embryos were cultured on medium supplemented with kinetin with or without coconut water. In this system, an average 67 microspores per responsive anther began cell division but only 3.69 embryos were formed per responsive anther after 6 wk. Adventitious embryos could be induced if the embryos, once formed, remained on initiation medium for 10 wk instead of being transferred to regeneration medium. Developmental stages which may be amenable to changes that could enhance plant production were identified. The potential to use this information to enhance plant production is discussed.

Key words: embryogenesis; starch medium; *Triticum aestivum* L.

INTRODUCTION

Plant regeneration, regardless of ploidy level occurs by either somatic embryogenesis or organogenesis. In wheat (*Triticum aestivum* L.) haploid plants have been regenerated via organogenesis by callus formation (Schaeffer et al., 1979; de Buyser and Henry, 1980; Wei, 1982; Ouyang et al., 1983; He and Ouyang, 1984; Lazar et al., 1985; Huang, 1987; Szakacs et al., 1988; Feng and Ouyang, 1989; Zhou and Konzak, 1989). Haploid plants also have been regenerated via embryogenesis of microspore-derived calluses (Armstrong et al., 1987) and direct microspore embryogenesis on solid (agar) medium (Anderson et al., 1987; Jones and Petolino, 1987; Datta and Wenzel, 1987; Sagi and Barnabas, 1989) and on liquid medium (Henry and de Buyser, 1981; Armstrong et al., 1987; Chu and Hill, 1988; Jones and Petolino, 1988) supplemented with glucose, fructose, and mannitol (Chu et al., 1990) or supplemented with maltose (Last and Brettell, 1990; Orshinsky et al., 1990). Haploid plants also have been regenerated via embryogenesis from haploid calluses derived from cultured young inflorescences of wheat. These calluses retained their ability for high frequency plant regeneration after 1.5 yr (Chu et al., 1987). Although the number of calluses or embryos initiated and the number of plants regenerated have been carefully described, the above studies only briefly describe the development of calluses and embryos to indicate organogenesis or embryogenesis. Because none of the above studies describe organogenesis or embryogenesis in detail, a comprehensive description of the events leading to callus or embryo, and plant formation is needed to identify potential stages for improving wheat anther culture.

Much of the previous research to improve wheat anther culture methodology has concentrated on media modifications. These media include potato (Marsolais et al., 1984; Henry and Buyser, 1985; Lazar et al., 1985; Marburger et al., 1987; Armstrong et al., 1987), N6 (Chu and Hill, 1988; Chu et al., 1990), and 85D12 medium (Liang et al., 1987) with various modifications. However, yields of haploid plants are still relatively low in comparison to the total number of microspores cultured and it is obviously possible to make further improvements in anther culture protocols. Hence, the objective of this research was to quantify and describe in detail microspore development leading to the formation of multicellular structures and embryos capable of regenerating plants. Careful attention was given to the consecutive stages of embryo development (e.g., cell division, suspensor formation, embryo polarization, scutellum, and organ primordia differentiation). The potential to use this information to enhance plant production will be discussed.

MATERIALS AND METHODS

Plant material. Plants of spring wheat (*Triticum aestivum* L.) cv. 'Pavon 76' were grown under controlled environmental conditions as previously described (Yuan et al., 1990). Tillers with anthers containing uninucleate microspores were selected, the leaves were removed, and the tillers were sterilized in a 20% solution of commercial bleach (5% NaOCl) for 20 min with periodic agitation. After three washings with sterile distilled water,

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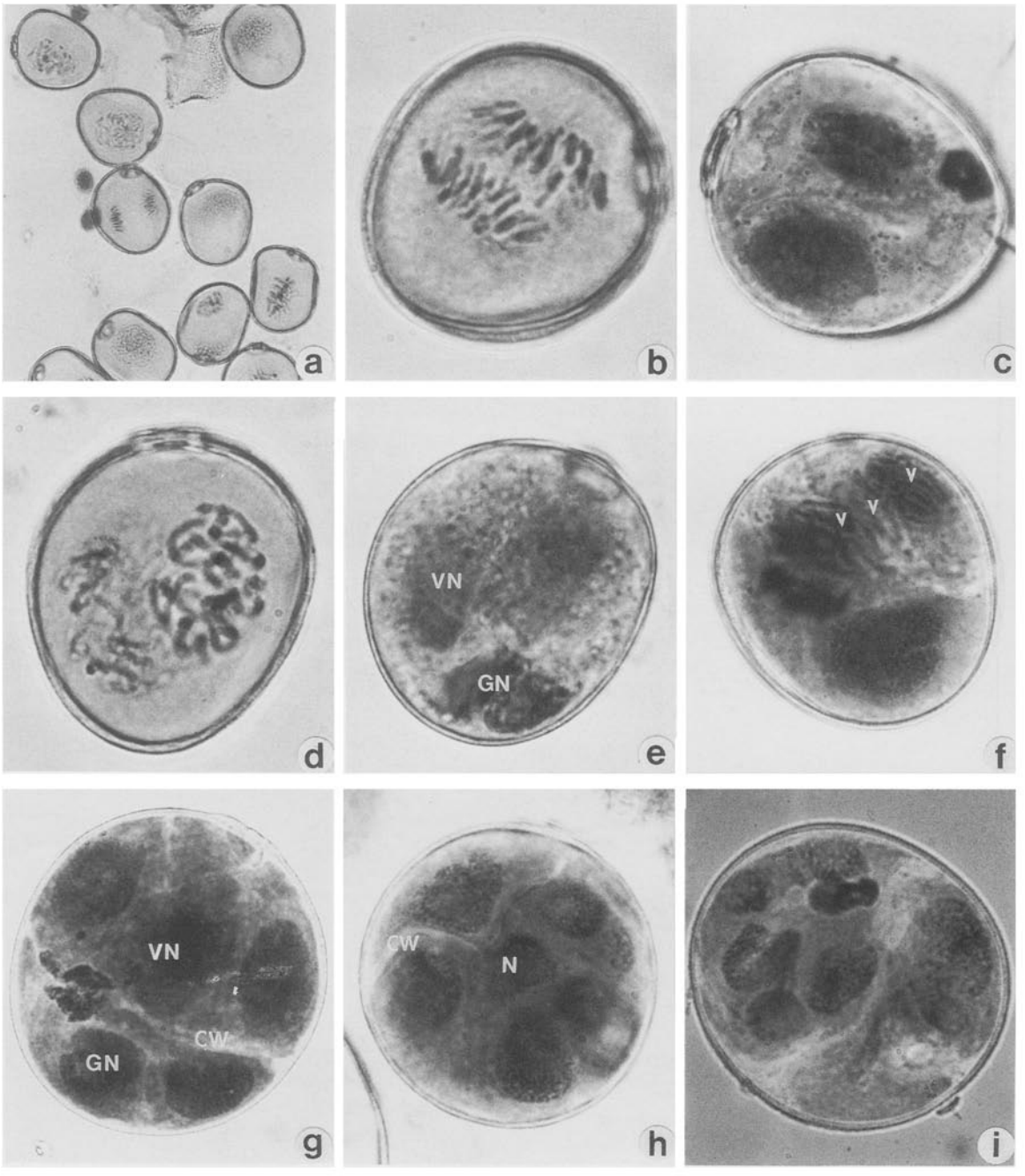

FIG. 1. Initial microspore cell divisions leading to multicellular structures on modified 85D12 starch medium (*GN* = generative nucleus, *VN* = vegetative nucleus, *N* = nucleus, *CW* = cell wall). ×400 or ×200. *a*, Numerous responsive microspores inside anther after 48 h. *b*, First metaphase leading to the two nuclei microspore stage. *c*, Microspore with two not equal nuclei. *d*, Synchronized second division of microspore nuclei. *Note* different size of metaphase chromosomes. *e*, Microspore with four nuclei microspore after 5 days in culture. *Note* different size of nuclei. Use of light microscopy did not allow identification of possible cell wall formation at this stage. *f*, Synchronized division of three vegetative nuclei (*arrow*) in multicellular structure. Initial stage of cell wall formation was visible using light microscopy. *g*, The separation of vegetative nuclei from two generative nuclei by the cell wall after 7 days in culture. *h*, Multicellular structure with well-formed cell walls. *i*, Multicellular structure after 10 days in culture.

spikes were dissected from the tillers and the youngest spikelets (at the bottom and top of the spike) were discarded. The selected anthers originated from the six to seven middle spikelets on each side of the rachis and 30 anthers were plated per petri dish (6 cm diameter). The petri dishes contained a 85D12 medium (Liang et al., 1987) modified to include 9.1 μM 2,4-dichlorophenoxyacetic acid, 5.4 μM napthaleneacetic acid, 1.2 mM asparagine, 3.4 mM glutamine, and 400 μM each of the following amino acids: alanine, arginine, aspartic acid, lysine, proline, and serine. The medium used 5% wheat starch (Aytex-p, Ogilvie Mills, Englewood Cliffs, NJ) for a gelling agent. This medium was made by adding filter-steri-

lized mineral salts, vitamins, amino acids, plant growth regulators, and sucrose to autoclaved wheat starch which was dispersed in liquid. Embryos were cultured on germination medium containing Murashige-Skoog (1962) salts, supplemented with 3% sucrose, 4.6 μM kinetin with or without 5% coconut water (vol/vol), 0.6% agar (wt/vol), and adjusted to pH 5.8 before autoclaving.

Tissue culture material. To study microspore development and formation of multicellular structures and embryos, 30 to 60 anthers per sampling time were sampled 24, 48, 72 h, 5, 7, 10, 13, 14, 17, 19, 20, 21, and 28 days after being placed on initiation medium. To further understand and determine the frequency of embryo formation, a second anther culture experiment having five culture replications in time (total of 2369 anthers, Table 1) were examined for morphogenic characteristics at 7-day intervals from Day 21 to Day 70 in culture. All anther culture experiments were initiated between August and November 1990.

Light and scanning electron microscope examination. In the first experiment, the sampled anthers were fixed in foremaldehyde:acetic acid:alcohol, 1:1:3 (FAA) solution for 24 h and then stored in 70% ethanol. Before staining with 1% acetocarmine for 1 h, anthers from the later sampling dates were hydrolyzed in 1.0 *M* HCl for 20 min at 60° C and washed in distilled water. For these cytological analyses using a light microscope, 550 anthers were used.

In the second experiment, the number of embryos was determined after 6 wk of culture using a dissecting light microscope. To describe the developmental stages of microspore-derived embryo and adventitious embryo formation, some explants were examined using scanning electron microscopy. Anthers at the appropriate stage of development (35–70 days in the initiation medium) were selected and analyzed. Selected material was fixed by using either FAA or glutaraldehyde (2.5% glutaraldehyde in 0.1 *M* Sorenson's phosphate buffer, pH 6.8) fixatives. For dehydration, the samples were placed in microporous specimen capsules and dried with CO_2 liquid in a critical point dryer type Samdri PVT3. The samples were coated with gold-palladium using a Sputter Coater EBTEC. Finally, samples were scanned using a scanning electron microscope AMRAY model 1000-A.

Results and Discussion

Each of the selected anthers contained approximately 1200 uninucleate microspores in the proper stage for initiating cell division. Of the 550 anthers cumulatively used to analyze microspore development, 183 (33.3%) possessed responsive microspores as determined by cell division and differentiation of multicellular structure. The number of responsive microspores varied from anther to anther, and the stage was not synchronized within an anther.

The first step in morphogenesis (Figs. 1 *a–c*) was the division of the microspore nucleus into two nuclei that were unequal in size. In responsive anthers, 31% of the microspores had nuclei that divided. From our data using light microscopy we were unable to determine if a thin cell wall formed after this nuclear division, hence we are not sure if there were multinuclear or multicellular structures. After 48 h in culture, the first nuclear division was recognized (Fig. 1 *a*). At 72 h, additional microspores underwent division, including some undergoing the second mitotic division (Fig. 2). At 5 days in culture, the most advanced microspores had undergone divisions leading to four nuclei (cells, Fig. 1 *b–e*). At 7 days in culture, multicellular structures with up to 10 nuclei had formed. The initial stages of microspore response (7 days) is 16% of the average time in culture required under our conditions to obtain fully formed embryos (42 days). Our results are different from the data presented by Henry et al. (1984) who reported that after 7 days in culture, a large number of surviving microspores in anther culture were still in the uni- or binucleate stage. By this time in our culture, the initial stage of microspore embryogenesis was completed with the formation of multicellular structures.

TABLE 1

ANTHER RESPONSE AND FREQUENCY OF EMBRYO FORMATION OF *TRITICUM AESTIVUM* L. cv. PAVON 76 AFTER 6 WK IN CULTURE ON STARCH 85D12 MODIFIED MEDIUM FOR FIVE SEPARATE CULTURE REPLICATIONS

Replication	Anthers			Embryos		
	Cultured No.	Responsive		No.	Per Responsive Anther	Per Cultured Anther
		No.	Percent			
1	449	102	22.7	396	3.88	0.88
2	420	43	10.2	150	3.48	0.35
3	435	59	13.6	178	3.01	0.40
4	285	47	16.5	187	3.97	0.65
5	780	148	19.0	564	3.81	0.72
Total	2369	399	16.7	1475	3.69	0.62

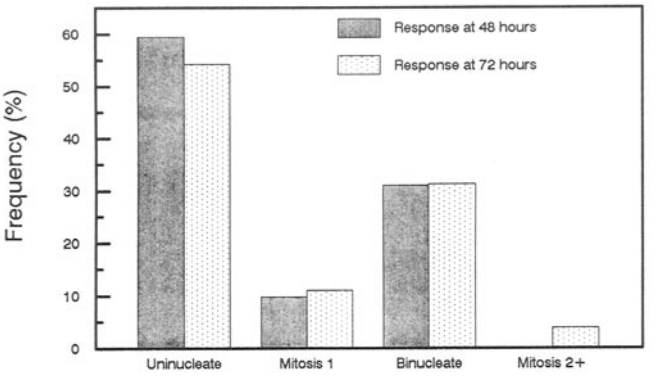

FIG. 2. Distribution (%) of uninucleate, first mitosis, binucleate, second mitosis, and more developed microspores of *Triticum aestivum* L. cv. Pavon 76 cultured on starch 85D12 modified medium after 48 and 72 h.

Between 7 and 21 days in culture, multicellular structures increased in cell number (the cell wall was clearly visible using light microscopy). By Day 9 in culture the majority of the multicellular structures had not less than 4 and up to 10 cells (Fig. 1 *e–h*). A dynamic increase in cell number (Fig. 1 *i*) occurs and leads to the formation of a globular embryo between 13 and 19 days in culture (Fig. 3 *a,b*). The embryos are characterized by the formation of the epidermis. The presence of the epidermis may be the first visual sign of differentiation events leading to the development of embryos. As cell number and the size of the embryos increased (Fig. 3 *c–e*), the globular embryos broke through the anther wall (17 days after culture, 41% of culture time; Fig. 3 *f*) and could be shed from the anther onto the medium. Late globular embryos can be characterized by a suspensorlike structure (Fig. 3 *d,e,g*). Schaeffer et al. (1979) also observed haploid embryonic structures (now recognized as having suspensorlike structures) of cv. Centurk after 21 days in culture. Sunderland et al. (1979) similarly found in barley that suspensorlike structures are formed early in morphogenesis. The suspensorlike structures came from cells that were determined at a very early stage of barley microspore-derived multicellular structure development. In wheat, previous work looking only at the early stages of morphogenesis with cv. Chris cultured on potato medium reported the direct formation of proembryonic structures.

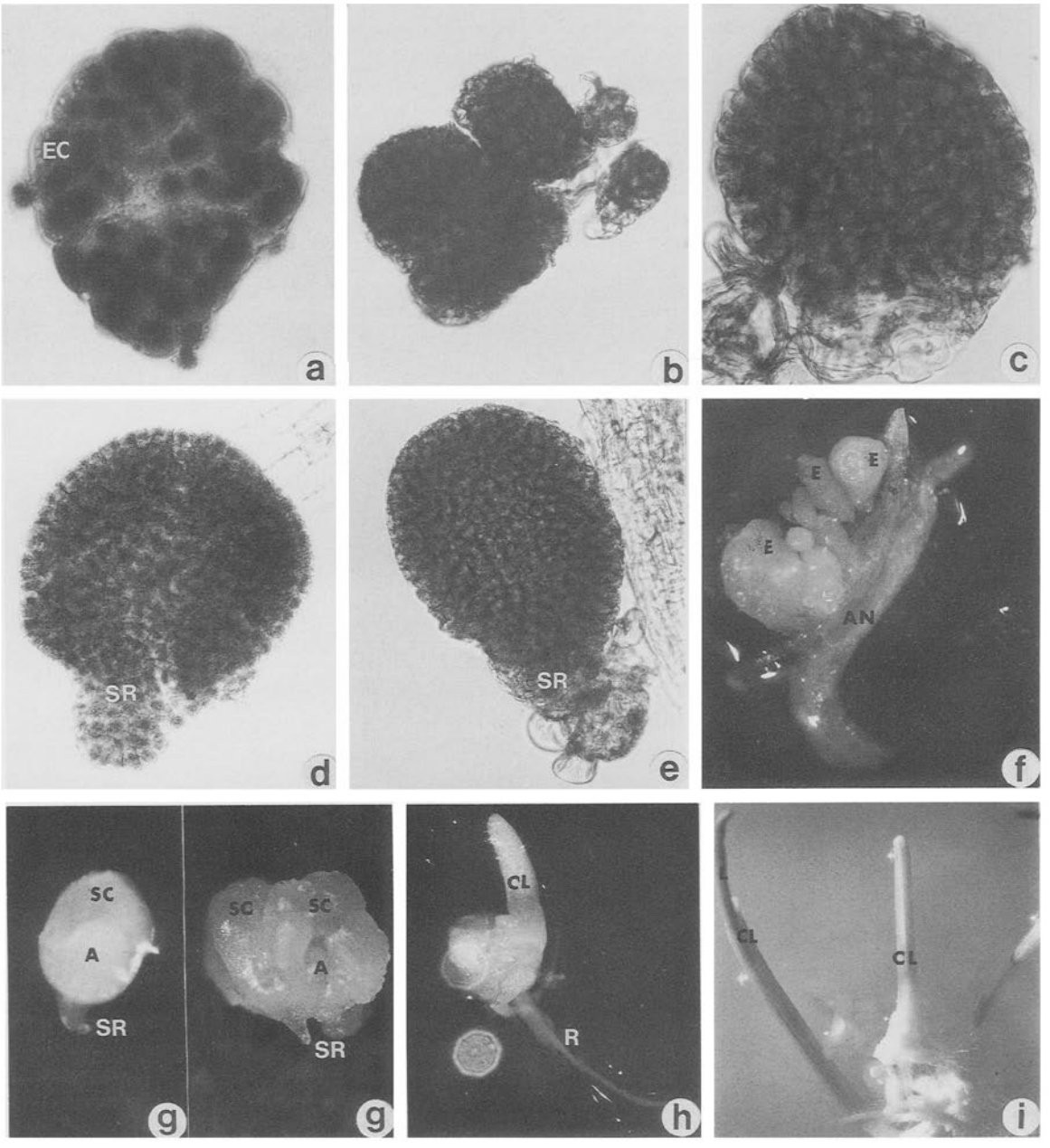

FIG. 3. Microspore-derived embryo differentiation on modified 85D12 starch medium (*A* = axis of embryo, *AN* = anther locule, *CL* = coleoptile, *E* = microspore-derived embryo, *EC* = epidermal cell, *L* = leaf, *R* = root, *SC* = scutellum, *SR* = suspensor). *a–e* ×120; *f,g* ×30; *h,i* ×20. *a*, Multicellular structure. *Note* epidermal cells; *b*, four embryos at different stages of development after 21 days in culture; *c*, globular embryo; *d*, globular embryo with developed suspensor; *e*, elongated embryo with distinct suspensor; *f*, anther locule with embryos in various stages of development; *g*, *left*, fully developed embryo with scutellum, axis, and distinct suspensor, *right*, twin embryo growing with thick scutellum, *Note* embryo axis and suspensor; *h*, germination of embryo; *i*, coleoptile and green leaf of plantlets.

The research did not sample later stages that could indicate embryogenesis. These proembryonic structures were believed to be capable of forming either embryos or morphogenic callus (Hassawi et al., 1990).

In zygotic embryogenesis, the first mitotic division determines the function of the derivative cells. One of the cells develops into a globular embryo, and the second one develops into a suspensor. In case of wheat microspores cultured in anthers, we could not confirm this early determination. However Fig. 1 *f* shows independent synchronized divisions of some nuclei. This stage is comparable to the cell suspensor determination stage in multicellular structures of barley microspores described by Sunderland et al. (1979). The absence of a suspensor determination stage may lead to callus formation, as opposed to embryo formation.

The presence of a suspensorlike structure also indicated that the development of microspore-derived embryos was similar to the zygotic embryo developmental pattern. Occasionally, embryo structures without a fully developed suspensorlike structure were observed. The presence of suspensorlike structures may be the second visual marker indicating a microspore-derived multicellular structure developing into an embryo.

In responsive anthers, embryo development was not synchronized for all multicellular structures at 21 days in culture. In a single anther, multicellular structures and embryos in various stages of embryogenesis could be identified (Figs. 3 *f* and 4, *a*). The number of advanced multicellular structures and embryos varied from 1 to 262 per responsive anther.

The next stage (28 days in culture; 67% of time in culture) was characterized by embryo elongation and polarization in which shoot meristem organization had occurred. Scutellar initial cell determination also was recognized (Fig. 4 *c*). Meristem organization and scutellum formation are the consequtive and important stages of normal embryo differentiation. The next stage of development (35 days in culture, 83% of time in culture) was characterized by the presence of a few fully formed single embryos (Ozias-Akins and Vasil, 1982; Magnusson and Bornman, 1985) (Figs. 3 *g left* and 4 *d*) and twins that had two scutellums each with an embryo axis and commonly ended with a suspensor (Figs. 3 *g right* and 4 *e*). Other researchers also identified later stages of morphogenesis. Jones and Petolino (1987) found embryolike structures, being white and globular in appearance, of soft wheat cultivars after 28 days in culture. Chu and Hill (1988), using modified N6 medium, reported with little experimental detail that the development of embryos from microspores was similar to zygotic embryogenesis during 10 to 30 days of culture. Embryo formation can be affected by media constituents. Media with glucose or monosaccharides stimulated the formation of scutellumlike structures after 25 days (Chu et al., 1990).

After 6 wk (100% of culture time) numerous embryos were completely developed and were similar to zygotic embryos (Figs. 3 *g left* and 4 *d*). A few embryos completed embryogenesis in 4 wk, which is similar to the time needed to complete the zygotic pathway and similar to the results described for somatic embryogenesis in diploid embryogenic wheat cultures (Ozias-Akins and Vasil, 1982; Magnusson and Bornman, 1985). In our study, embryos transferred to regeneration medium supplemented with kinetin with or without coconut water easily germinated showing distinct coleoptile and radicle elongation (Fig. 3 *h,i*). This result further confirms embryogenesis.

The number of responsive anthers and the number of morphogenic microspores or derivatives decreased with time in culture. In experiment 1 with early sampling times, 33.3% of the anthers were responsive. In experiment 2 with sampling at 42 days in culture, only 16.7% of the anthers were considered as being responsive (Table 1). In comparison to the 1 to 262 multicellular structures and embryos that were counted in responsive anthers at 21 days in culture, by 42 days in culture the final number of developed embryos varied from 1 to 21 per responsive anther (distribution given in Fig. 5). The majority of responsive anthers (30%) had only one embryo. Responsive anthers with 11 to 21 embryos were the least frequent. The average number of embryos per responsive anther

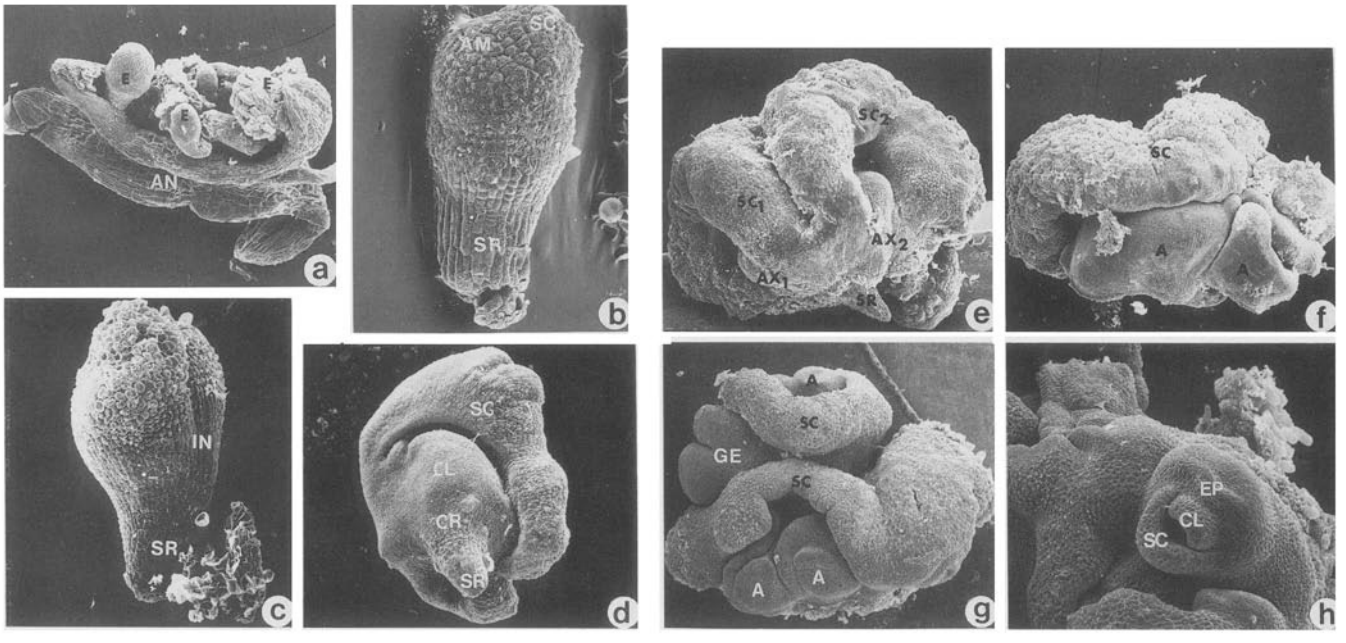

FIG. 4. Microspore-derived and adventitious embryogenesis in anther culture on modified 85D12 starch medium (*A* = axis, *AX1* and *AX2* = axis of twin embryo, *AM* = region of apical meristem formation, *AN* = anther, *CL* = coleoptile, *CR* = coleorhiza, *E* = microspore-derived embryo, *EP* = epiblast, *GE* = globular adventitious embryo, *IN* = indentation, *SC* = scutellum, *SR* = suspensor). Scanning electron microscopy from ×23 to ×120. *a*, Open anther sack with multiple embryos in various stages of development; *b*, apical meristem sphere and initial scutellum stage differentiation after 21 days in culture; *c*, initial stage of twin embryo, *Note* formation of indentation between twins; *d*, embryo with scutellum, coleoptile, and coleorhiza after 28 days in culture; *e*, twin embryo with overgrowing scutellum after 42 days in culture; *f*, embryogenic callus formation on scutellum of microspore-derived embryo after 56 days in culture; *g*, adventitious multiembryo structure after 70 days in culture; *h*, adventitious embryo in epiblast and coleoptile differentiation stage.

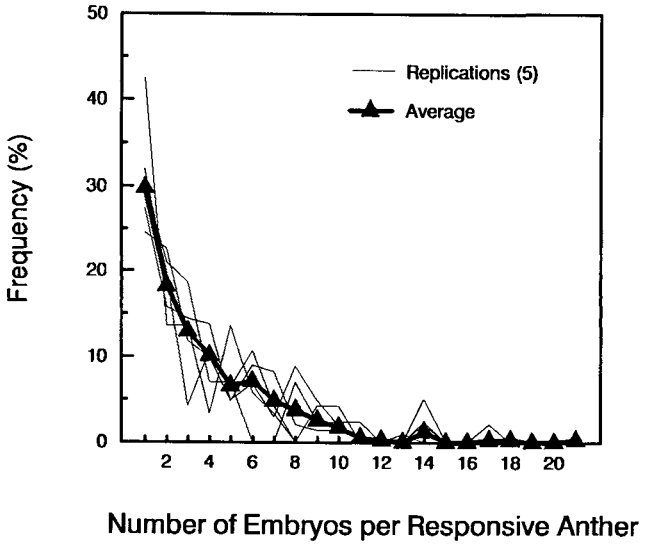

FIG. 5. The frequency of the number of embryos per responsive anther formed by *Triticum aestivum* L. cv. Pavon 76 on starch 85D12 modified medium after 6 wk of the culture.

was 3.69 (Table 1). This average was obtained from five culture replications conducted over a 4-mo. period. Each replication had similar results indicating the repeatability of the anther culture technique. Compared to results reported for embryo production with alternative systems, this system is competitive to those based on liquid medium with or without using monosaccharides (Henry and de Buyser, 1981; Chu and Hill, 1988; Chu et al., 1990; Last and Brettel, 1990) in terms of embryos per 100 anthers.

In extended cultures (70 days) on initiation medium, the microspore-derived embryos formed embryogenic callus on their scutellum and epiblast. Adventitious embryos formed on these structures displayed most of the stages of embryogenesis (Fig. 4 *g,h*), including a well-developed scutellum and axis. Multiaxis structures also were formed.

On the basis of these results, it is clear that our microspore embryogenesis system can be improved. The majority of the anthers were unresponsive (Table 1). In responsive anthers, which contained approximately 1200 microspores per anther, an average of 67 microspores went through cell division. This number is only 5% of the total microspores in an anther and 25% of the number of microspores (262) that formed multicellular structures and embryos in our most responsive anther. Finally, from the 67 microspores per responsive anther that began embryogenesis, we obtained on average only 3.69 well-formed embryos per responsive anther (0.62 embryo per plated anther).

Improvements in this anther culture system could be made by increasing the number of responding anthers. This may be done by more carefully identifying anthers at the best stage for culture. Improvements could also be made by identifying media components that synchronize cultures passing through consecutive stages of embryogenesis (initial cell division, globular stage, suspensorlike structure stage, meristem organization with scutellum elongation stage, and shoot and root primordia differentiation stage) which would allow more effective media manipulations. In our research, the asynchronous development of embryos may hinder the development of better anther culture systems. Specifically, the asynchronous development of embryos prevents developing an optimized medium for a specific embryogenic stage. Our using a medium which allowed all embryogenic stages to continue to develop may not have been optimal for an individual stage. This could explain the loss of potential embryos at every embryogenic stage. Methods to separate multicellular structures and embryos into uniform embryogenic stages would be beneficial for future studies of factors affecting embryogenesis.

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