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# Developmental Dynamics of Wheat (*Triticum aestivum* L.) Microspores under Culture

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## Abstract

**Doubled haploid production via microspore culture is a technique known to accelerate crop breeding by shortening the breeding cycle through achieving homozygosity in one generation. Prior research observed that some embryogenic microspores aborted their development before reaching the embryoid stage. Such embryogenic abortion reduces embryoid yield, making microspore cultures less efficient. The present research aims at identifying stages during which microspore development is susceptible to embryogenic abortion. Information gained through delineation of the developmental dynamics of microspores in culture could be used to improve the efficiency of microspore culture. Embryogenic microspores were isolated from stress-treated wheat (*Triticum aestivum* L.) tillers and cultured in liquid medium. The development of embryogenic microspores was monitored over a 35 day period. At day 7, 10, 14, 21, 28, and 35, the developing microspores were counted and categorized into multicellular structures, pre-embryoids, immature embryoids and mature embryoids. The results showed that 44% - 62% of embryogenic microspores halted their development before the mature embryoid stage. Of these aborted embryogenic microspores, 21% - 33% terminated as multicellular structures, 16% - 19% arrested their development as pre-embryoids, and 7% - 10% halted development as immature embryoids. Identifying factors that are responsible for embryogenic abortion and finding remedy to the issue will help improve the efficiency of doubled haploid production.**

## Keywords

**Microspore Culture, Embryogenic Abortion, Doubled Haploids, *Triticum aestivum* L.**

## 1. Introduction

*Triticum aestivum* was once considered as a recalcitrant species for androgenesis—a process through which

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plants are regenerated from anthers or immature pollen. Although the first success in androgenesis was achieved half a century ago with *Datura inoxia* [1], methods for mechanical isolation and culture of microspores for plant regeneration were developed much more recently in bread wheat [2]-[6]. One of the distinctive advantages with the more recent culture systems is the production of plants from induced microspores through *in vitro* embryogenesis. The culture of isolated microspores not only increases yields of double haploid plants, but also affords opportunities for basic research concerning *in vitro* embryogenesis.

Embryogenesis *in vivo* results in development of an embryo, a reproductive process that requires pollination and ensuing fertilization. However, *in vitro* embryogenesis, also called somatic embryogenesis, is the production of a pseudo-embryo (or embryoid) from a cell without involving fertilization. This embryoid so obtained is functionally equivalent to an embryo and able to germinate into a plant under adequate conditions. To that end, microspores must first be developmentally reprogrammed to produce embryoids [7]. The reprogramming is necessary since a microspore is innately programmed to differentiate into a highly specialized male gametophyte, a mature pollen grain. Reprogramming can be achieved by using artificial manipulation such as physical, physiological, and chemical treatment [8]. The most appropriate time to reprogram microspores for embryogenesis in cereal species is the mid to late uninucleate stage [4] [9]. During this period, microspores are not yet fully committed to their developmental fate and are thus susceptible to androgenic induction [8]. Once the first gametophytic mitosis occurs, the cytoplasm in immature pollen may be dominated with gametophytic cues, making it very difficult to reverse the development toward a male gametophyte [10] [11].

Various forms of pretreatment have been used to induce microspore embryogenesis. These include, but are not limited to, temperature shock, high osmolarity, reduction in sugar or nutrients, and exposure to ultraviolet light. Water stress, anaerobic conditions, and inducer chemicals were also employed for reprogramming (as reviewed in [8]). One of the most effective means of reprogramming is temperature stress. Heat shock at 33˚C was found to result in higher embryoid/callus yields, as well as a greater frequency of spontaneous chromosome doubling [4] [6] [12]. Once the microspores are reprogrammed for embryogenesis, they are released from the donor plant tillers and plated to a nutrient medium for cell divisions and subsequent differentiation into embryoids. In nutrient medium, embryogenic microspores progress through the developmental stages of multicellular structures, pre-embryoids, immature embryoids, and mature embryoids.

Various components are required to sustain the development towards mature embryoids from embryogenic microspores. These include balanced nutrients, optimal pH, adequate osmolarity and temperature. The inclusion of ovaries in culture was found to increase the number of pre-embryoids, embryoids, and regenerated plants [13] [14]. Ovaries serve to sustain the overall process of embryogenesis and to improve the quality of embryoids [14] [15]. In short, when adequate culture conditions are in place, embryogenic microspores will more likely develop into mature embryoids, which are able to germinate into intact plants.

While induced microspore embryogenesis seems to be the method of choice for doubled haploid production, challenges for generating a sufficient number of plants for practical breeding still remain. One of the challenges in wheat is the developmental abortion of initially embryogenic microspores during culture. A significant number of embryogenic microspores failed to evolve into mature embryoids, thus reducing the number of double haploids. Some embryogenic microspores started cell divisions but terminated at various points, as multicellular structures, pre-embryoids or immature embryoids. Although such embryogenic abortion has been observed in multiple species, a stage-wise quantitative analysis of embryogenic abortion is lacking. A better understanding of the developmental dynamics among embryogenic microspores is probably the first step towards finding a remedy for embryogenic abortion, hence allowing a wider use of this technology in wheat breeding. Thus, the present study is designed to answer two related questions: 1) how frequently do embryogenic abortions occur in culture? 2) at which stage of the embryogenic development do abortions occur the most? Once these two questions are answered, we can then move on to test if any of the developmental halts can be alleviated. The ultimate goal is to improve the efficiency of doubled haploid production through reducing the abortion frequency among embryogenic microspores.

## 2. Materials and Methods

### 2.1. Raising Microspore Donor Plants

Three genotypes were used for these experiments: Chris, Pavon 76 and WED202-16-2. Donor plants were grown in a plant growth chamber programmed on a daily cycle of 16 hours of light at 25˚C, followed by 8 hours

of dark period at 17˚C. Plants were watered as needed. This controlled environment minimized physiological stress on the developing microspores within anthers of the donor plants. Once microspores had reached the mid to late uninucleate stage, they were suitable for embryogenic induction. The tillers of each genotype were excised and prepared for stress treatment. Details for raising donor plants, sampling, and isolating microspores can be found elsewhere [4] [6] [16].

### 2.2. Microspore Culture and Data Collection

After isolation, embryogenic microspores were plated to a liquid medium for embryogenesis. The medium contained balanced nutrients, adequate osmolarity and wheat ovaries to allow cell divisions to proceed through a series of stages defined as multicellular structure, pre-embryoid, immature embryoid and mature embryoid. All microspores were cultured according to earlier publication [16]. Microspores of each wheat genotype were distributed into four Petri dishes containing growth medium and fresh ovaries, and observed for 35 days. At day 7, 10, 14, 21, 28, and 35, a sub-sample of developing microspores for each genotype were counted and categorized based on their developmental morphology, as illustrated later in the Results section. Since the number of microspores in each Petri dish exceeded five thousand, it was impractical to count and score them all. Therefore, three 8 mm diameter fields in each Petri dish were randomly selected for counting using Zeiss Axio Vert A1 inverted microscope. The results presented were a pooled mean of three fields from four replicas of each genotype. Developmental stage counts were recorded as a percentage based on the total number of microspores presented in each field.

## 3. Results

Embryogenic microspores, with a defined "star-like" structure reported earlier [6] [11], began the first cell divisions within 14 to 20 hours after isolation, and formed multicellular structures within a week (**Figure 1**). Pre-embryoids (**Figure 2**) began to emerge out of microspore cell walls within 10 to 14 days, immature embryoids (**Figure 3** and **Figure 4**) formed between 14 to 21 days, and mature embryoids (**Figure 5**) began to emerge at 28 days in culture. Once mature embryoids reached the size of 2 mm in diameter, they were transferred to a semi-solid medium for plant regeneration. Within 5 to 7 days following the transfer, green shoots (**Figure 6**) emerged. Ten to 14 days after the transfer, the plantlets were potted for continued growth and seed harvest [8] in the greenhouse. These developmental stages in microspore embryogenesis served as important landmarks for our quantitative study of the embryogenic abortions.

The developmental dynamics of microspores isolated from Chris are shown in **Figure 7**. At day 7, only MCS appeared and accounted for 28.3% of the total microspores. That percentage increased slightly to 29% at day 10, while 6% pre-embryoids were recorded on the same day. At day 14, the MCS declined sharply to 10.4% while pre-embryoids rose to 29.5%. The earliest immature embryoids began to emerge at day 14, accounting to only 1.7%. At day 21, 28 and 35, MCS accounted for 9%, 9.1%, 8.2%, indicating insignificant change in MCS from 21 days onward. At day 21, pre-embryoids declined sharply to 7%, but immature embryoids reached their peak at 21.5%, only 1.1% developed into mature embryoids. From day 21 onward, pre-embryoids remained around 7%,

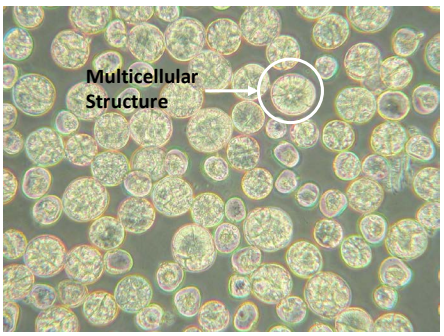


**Figure 1.** Multicellular structures at day 7 in induction culture, showing that all cells are still enclosed in a common microspore wall.

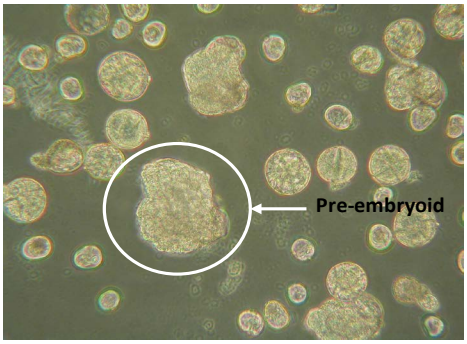


**Figure 2.** Photo at day 10 in induction culture, showing the rupture of microspore wall and emergence of pre-embryoids.

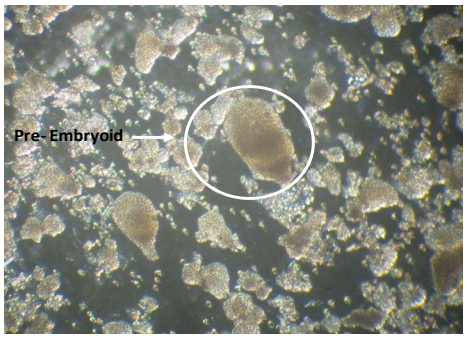


**Figure 3.** Photo at day 14 showing pre-embryoids in induction culture at more advanced stage.

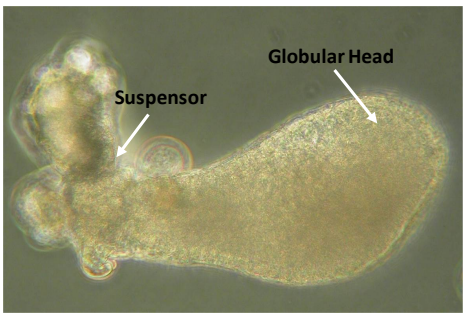


**Figure 4.** An **i**mmature embryoid at day 21 in induction culture showing the well-developed globular head and the suspensor.

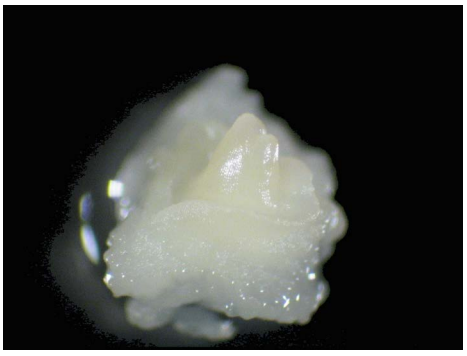

**Figure 5.** A mature embryoid at day 35 in induction culture showing the well-developed shoot axis.

**Figure 6.** A plantlet formed in 7 days after a mature embryoid was plated onto the regeneration medium.

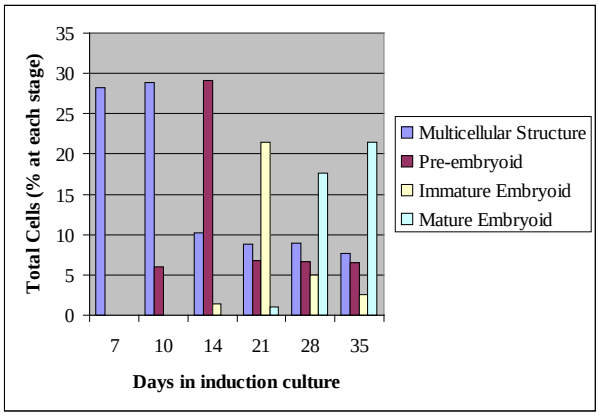


**Figure 7.** Developmental dynamics of microspores isolated from Chris over a 35 day period as microspores progressed from multicellular structures to mature embryoids.

representing the portion that was unlikely to advance beyond this stage. At day 28, immature embryoids declined sharply to 5% while mature embryoids rose to 17.5%. At day 35, only 2.5% remained as immature embryoids, while 21.7% have progressed to mature embryoids.

At day 35, total microspores consisted of 7.9% MCS, 7.1% pre-embryoids, 2.5% immature embryoids, 21.7% mature embryoids and 60.8% non-dividing microspores. An average of 17.5% dividing microspores failed to reach the finishing line as mature embryoids. In other words, 44.6% of dividing microspores aborted their development as MCS, pre-embryoids or immature embryoids. Among the 60.8% of microspores that failed to evolve into MCS, most never initiated cell division, and only less than 10% completed one cell division (visual estimate, not accurate counting). This largest fraction of microspores are unlikely the target for any medium manipulation for improving the efficiency of microspore culture. On the other hand, once embryogenic microspores advanced to immature embryoids, very few (<2.5%) aborted their development, as even more immature embryoids will grow into mature ones beyond day 35.

The developmental dynamics of microspores from Pavon 76 and WED202-16-2 are summarized in **Figure 8** and **Figure 9**. Although numbers at each data point vary slightly, the developmental pattern was almost identical to that of Chris. The MCS, pre-embryoids, immature embryoids and mature embryoids showed very similar rise and fall on day 7, 10, 14, 21, 28 and 35. At day 35, 7% MCS, 7.2% pre-embryoids, 3.5% immature embryoids, and 21% mature embryoids were observed for Pavon 76, while 8% MCS, 4.5% pre-embryoids, 3.7% immature embryoids, and 23% mature embryoids were recorded for WED202. Once again, approximately 60% of the microspores failed to develop into MCS; a very small percentage (3.5 and 3.7%) of developing microspores remained as immature embryoids at day 35. A total (MCS, pre-embryoids and immature embryoids) of more than 40% (45.7% and 41.3% for Pavon 76 and WED 202 respectively) of the dividing microspores aborted before reaching the mature embryoid stage.

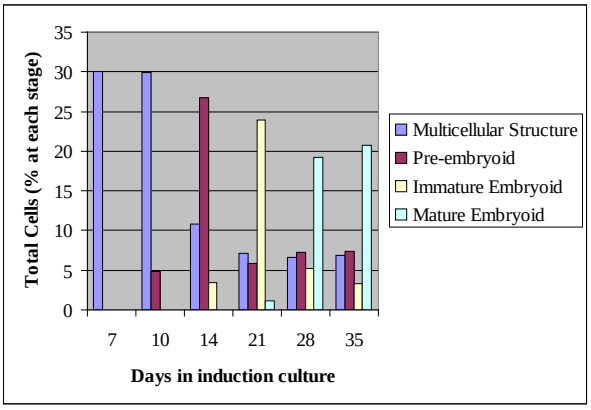


**Figure 8.** Developmental dynamics of microspores isolated from Pavon 76 over a 35 day period as microspores progressed from multicellular structures to mature embryoids.

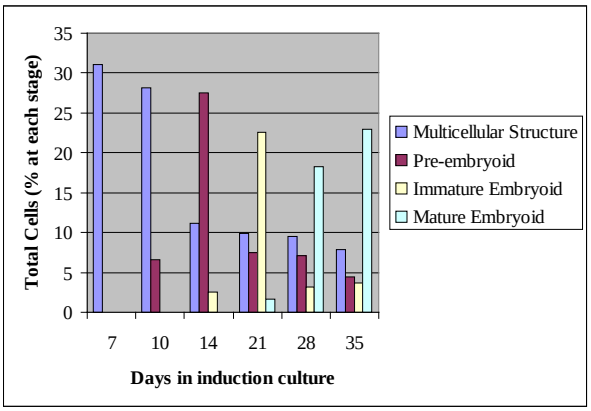


**Figure 9.** Developmental dynamics of microspores isolated from WED202-16-2 over a 35 day period as microspores progressed from multicellular structures to mature embryoids.

## 4. Discussion

The results indicate that of the total number of microspores initially cultured, about 40% proceeded to the multicellular structure phase while the remaining 60% failed to divide or divide beyond the first two cell cycles (**Figures 7-9**). The 60% non-dividing fraction likely represented microspores which were either non-embryogenic ones incapable of dividing, or embryogenic ones that had incurred substantial damage during blending, filtration and centrifugation. These damages to microspores were not visible through observation using an inverted microscope. Of the embryogenic microspores that proceeded to multicellular structures, 54% - 58% developed into mature embryoids for the genotypes studied. The remaining 42% - 46% halted their development at the multicellular stage (18% - 20%), pre-embryoid (11.5% - 18.6%), or immature embryoid (6.4% - 9.5%).

As more dividing microspores were observed to arrest their development as MCS or pre-embryoids (29.5% - 38.6%), the first 10 to 14 days in induction culture should be the target period for reducing the embryogenic abortion. Regardless of genotypes, the percentages of the multicellular structure and the pre-embryoid remained relatively constant for the day 21 onward. This consistency was also observed in all four Petri dishes of each genotype. These results indicate that after three weeks in induction culture, a vast majority of the dividing microspores that remained as multicellular structures and pre-embryoids will not develop any further, thus failing to reach the mature embryoid stage. By day 35 in induction culture, an average of 20% of the multicellular structures failed to develop into a pre-embryoid and 17% of pre-embryoids could not develop into immature embryoids. Therefore, after 21 days in induction culture a total of 37% of the multicellular structures observed

on day 10 halted their development. By day 28, microspores that developed into immature embryoids had a very high success rate of developing into mature embryoids. Of all microspores proceeded to immature embryoids, only 7% failed to evolve into mature embryoid, likely due to intensified competition for space and/or nutrients among emerging embryoids. So the goal for any future remedy for embryogenic abortion should be set to increase the number or percentage of immature embryoids, which corresponds quite well with the yield of mature embryoids.

Knowing at what stage in induction culture embryogenic microspores abort their development is valuable for several reasons. First, if abortion is caused by damage during pretreatment and isolation or other undue stress before the embryogenic culture, we can work to refine the protocol and minimize such damage and stress. The fact that at least 60% of microspores failed to divide and form MCS suggests that physical damage and other stresses before induction culture are significant impediments to microspore cultures. Further refinement of protocols in isolating and handling microspores might lower the embryogenic abortion. Secondly, knowing the detriment of wounding and/or stress damages to microspores may lead to a remedy that minimizes its impact. Nursing factors released into the ovary conditioned medium [6] or Arabinogalactan Larcoll [17] may be employed to help some microspores recover from such damages. These recovered microspores may exhibit a greater potential for development into mature embryoids than those otherwise destined for embryogenic abortion. Third, if embryogenic abortion occurs mostly after microspores start dividing, then we can conduct further research to optimize culture regime to reduce the abortion rate.

Our results seemed to suggest that embryogenic abortion could result from both pre-culture damage and initial culture stresses, as many seemingly embryogenic microspores failed to form MCS, and a considerable faction of MCS stopped developing further along embryogenesis. To minimize the pre-culture damages, one might consider shedding microspores from excised anthers [9] [18] [19] rather than mechanical blending. Although useful especially when the donor spikes are limited for a small breeding population, the shedding technique does require additional time for excising and plating anthers. When there are enough donor spikes, mechanical isolation would still be the method of choice due to its simplicity, speed and capacity to process large number of samples or breeding lines within a short period of time [4] [16].

Recent research found that Arabinogalactan Larcoll, a sugar, was useful for decreasing the mortality rate of developing microspores. Results suggest that Arabinogalactan Larcoll aids in microspore recovery from pretreatment and isolation stress [17]. Our study found that 60% of microspores were unable to divide, at least partially due to damages during these processes. By repairing some wounds/damages, the percentage of healthy microspores would increase, which would improve the probability of developing embryogenic microspores into mature embryoids. Overall, Letarte's study found that there was a fivefold decrease in the frequency of dead microspores. Not only were embryogenic abortions decreased, but cellular divisions occurred more rapidly [17].

There are several possible causes for the embryogenic abortions observed during microspore culture. One particular cause could be a competition for nutrients. At day 14 in induction culture, the number of cells that aborted was very high. This is also the time period where the immature embryoid increases in frequency. The immature embryoid is much larger than the multicellular structure and pre-embryoid. Not only is it made up of a larger number of cells, but these cells also begin to differentiate. The immature embryoids are increasing in size at a more rapid rate than the multicellular structures and pre-embryoids. Since they are larger and more specialized, it is possible that the immature embryoids are pooling more of the resources, leaving an insufficient amount for the other smaller structures. One possible solution to this problem could be to remove the immature embryoids from the induction culture at approximately day 21. They can then be placed in a separate Petri dish with growth medium, thus leaving the multicellular structures and pre-embryoids free to develop without having to compete with immature embryoids for nutrients.

Another possible cause resulting in spontaneous abortion is a competition for space. As the microspores progress through the various phases and cellular divisions occur, the population density within the Petri dish drastically increases. This puts added strain on the developing microspores and may halt development. As the cell frequency within the Petri dish increases, the larger structures also have a greater advantage over the less developed structures. These structures have already begun to differentiate and thus have a selective advantage over the smaller and less developed structures.

One possible solution to reduce culture stress could be to refresh the medium and add fresh ovaries to each Petri dish at day 21 in induction culture, as suggested by earlier reports [13]-[15]. However, the refreshing protocol does not address the competition for space or possibly oxygen, which leads to a third and better solution:

the combination of refreshing and transfer of multicellular structures and pre-embryoids to a separate Petri dish. In a preliminary experiment we used a 5 ml pipette to suck up most multicellular structures and pre-embryoids and transferred them in another Petri dish. Fresh medium was added to both the old and new plates. Almost all the immature embryoids evolved into mature embryoids and far less multicellular and pre-embryoids halted their development (data not shown due to insufficient replicates). More importantly, the quality of the mature embryoids seemed better, as measured by improved plant regeneration.

In conclusion, through analysis of the development dynamics of wheat microspores in culture, we find that many embryogenic microspores that initially divided into multicellular structures spontaneously aborted before completing their development into mature embryoids. These abortions occurred most frequently in the phases of multicellular structure and pre-embryoid before day 21 in induction culture. While prevention of embryogenic abortions is beyond the scope of this paper, there are many implications for future research. One future study could be to remove immature embryoids from the induction culture at approximately day 21 and test if more of the remaining multicellular structures/pre-embryoids will develop forward. The removal may leave the multicellular structures and pre-embryoids free to develop without having to compete with immature embryoids for nutrients and/or space. Another area of research could be to test the effects of refreshing the medium and adding fresh ovaries to each Petri dish at day 21 in induction culture. Furthermore, it might be worthwhile to explore the combination of refreshing and transferring of immature embryoids to a separate Petri dish. If the addition of ovary-conditioned medium and/or Arabinoglacta Larcoll to induction culture can reduce the minimal cell density required for embryogenic division, the competition for nutrients and/or space should not be an issue in the first place.

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## Abbreviation

MCS: multicellular structure.