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CELL BIOLOGY AND MORPHOGENESIS

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# Improving green plant production via isolated microspore culture in bread wheat (*Triticum aestivum* L.)

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**Abstract** Although a high efficiency of microspore embryogenesis can be achieved for a wide range of genotypes, some genotypes regenerate a high proportion of albino plants. Significant improvements in embryo regeneration and green plant formation were achieved by adding 10% NPB98 medium to a microspore pretreatment solution containing a chemical inducer formulation. For the wheat genotype WED 202-16-2, which is known to produce a moderate frequency of albinos, the plant regeneration rate was 15% higher, and the green plant percentage increased 27% following the addition of this nutrient supplement during embryo initiation. There were no negative effects on the responsive genotype Svilena. Embryo production in both genotypes was not affected. Our results indicate that providing readily available nutrients during microspore embryo initiation is effective in promoting plant regeneration and enhancing green plant development in a wheat genotype with a propensity for albinism.

**Keywords** Androgenesis · Albino · Doubled haploid · Nutrients · Plant regeneration

**Abbreviations** *DH*: Doubled haploid · *NPB*: Northwest Plant Breeding Company

## Introduction

An efficient doubled haploid technology would enable breeders to reduce the time and the cost of cultivar development relative to conventional breeding practices (Hu and Yang 1986; Konzak et al. 1987; Hu 1997). A highly efficient system for embryo production from isolated microspore cultures has been achieved by scientists and technologists at the Northwest Plant Breeding Company, and thousands of embryos can be obtained reliably from the microspores in a single wheat spike (Liu et al. 2001). While plant regeneration from microspore-derived embryos is not a problem for most genotypes, a high percentage of albino plants regenerate from some genotypes. The high efficiency of the NPB microspore culture system is attributed to a number of factors. One of the critical factors is believed to be a pretreatment containing a chemical inducer formulation, which results in a large population of embryogenic microspores. This unique treatment is a system in which microspores are provided with the chemical inducer formulation, the components of which are translocated from the formulated solution in the flask and tissues surrounding microspores, respectively, during the stress pretreatment (Liu et al. 2001). However, changes to these conditions decrease the embryo induction efficiency. Thus, with respect to total green plant production efficiency, a better strategy is to improve plant regeneration and increase the proportion of green plants among regenerants while maintaining the high efficiency for embryo production.

Genotypic differences in androgenesis induction do exist (Anderson et al. 1987; Tuvesson et al. 1989). Some genotypes naturally react better than others in response to environmental changes, such as stress treatments applied to microspores. To overcome such differences, we must provide a better environment to the microspores so that the physiological limitations can be partially compensated for with readily available nutrient resources. We hypothesize that genotypes with reduced plant regeneration and a high frequency of albino plant development are less competent in their efficiency to utilize the nutrients in tissues surrounding the microspores during the pretreatment of whole spikes. If this is so, then to reduce the number of albino plants that develop, an adequate supply of nutrients must be readily available to microspores during this pretreatment or “starvation” period. In other words, microspores should not be “starved” severely. In the NPB flask microspore culture system,

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nutrients in the flask should be readily available for transport via the vascular system of the stem to become available to the microspores. Thus, the addition to the flask solution of culture media that contain a chemical inducer formulation for inducing embryogenesis should provide a simple way to make a more balanced nutrient supply available to microspores, which then undergo initiation of microspore embryogenesis.

The investigation reported here was designed to determine if embryo quality – i.e. plant regeneration and formation of green versus albino plants – was affected by the supply of nutrients available to microspores during the pretreatment period when microspores were exposed to a chemical inducer formulation.

## Materials and methods

### Materials

Spring wheat line WED 202-16-2 (developed by Dr. Adriana Grama Dvosea, Volcani Institute, Bet Degan, Israel, and kindly made available to NPB) and the Bulgarian winter wheat Svilena (made available by the kindness of Kostadin Kostov of IWS, Dobruja, Bulgaria) were used. WED 202-16-2 is known to produce both green and albino plants, while Svilena produces a high proportion of green plants using the current NPB flask culture system. Methods for growing wheat plants, collecting tillers and microspore isolation have been described previously (Liu et al. 2001).

### Pretreatment of spikes

Three fresh tillers were placed in an autoclaved sterile flask containing 50 ml of sterile (autoclaved) distilled water and 0.01% (w/v) 2-hydroxy nicotinic acid (2HNA), 10 mg/l 2,4-dichlorophenoxyacetic acid, 2 mg/l 6-benzylaminopurine, 3 mg/l giberellic acid (the chemical inducer formulation), with or without 10% NPB98 induction medium; each flask was replicated twice. The full-strength NPB98 medium contains 232 mg $l^{-1}$ $(NH_4)_2SO_4$, 1,415 mg $l^{-1}$ $KNO_3$, 83 mg $l^{-1}$ $CaCl_2$-$2H_2O$, 200 mg $l^{-1}$ $KH_2PO_4$, 93 mg $l^{-1}$ $MgSO_4$-$7H_2O$, 5 mg $l^{-1}$ $H_3BO_3$, 0.0125 mg $l^{-1}$ $CoCl$-$6H_2O$, 0.0125 mg $l^{-1}$ $CuSO_4$-$5H_2O$, 0.4 mg $l^{-1}$ KI, 5 mg $l^{-1}$ $MnSO_4$-$4H_2O$, 0.0125 mg $l^{-1}$ $Na_2MoO_4$-$2H_2O$, 5 mg $l^{-1}$ $ZnSO_4$-$7H_2O$, 37.3 mg $l^{-1}$ $Na_2EDTA$, 27.8 mg $l^{-1}$ $FeSO_4$-$7H_2O$, 50 mg $l^{-1}$ myo-inositol, 0.5 mg $l^{-1}$ nicotinic acid, 0.5 mg $l^{-1}$ pyridoxine-HCl, 5 mg $l^{-1}$ thiamine-HCl, 500 mg $l^{-1}$ glutamine, 1 mg $l^{-1}$ phenylacetic acid, and 9% (w/v) maltose, adjusted to pH 7 and filter-sterilized. The open end of a sterile plastic bag (thin-walled, transparent grocery store vegetable bag) was then placed over the wheat tillers and the bag wrapped around the neck of the flask and sealed around the neck with masking tape to prevent microbial contamination and excessive loss of water. The flask was then placed in an incubator maintained at the dark setting at 33°C for 69 h. After the treatment, the embryogenic microspores typically had eight or more small vacuoles immediately enclosed by the cell wall. These vacuoles surround the condensed cytoplasm in the center, forming a fibrillar structure that can be observed under phase contrast in a Zeiss inverted microscope (Axiovert 25) by squashing an anther from the middle section of a treated spike (Liu et al. 2001).

### Culture of isolated microspores

Microspores were isolated separately for each replication with three spikes. Isolated microspores were cultured in liquid NPB 98 medium. An aliquot of 5 ml media per 60 mm×15 mm petri dish, at a density of approximately $3\times10^4$ microspores per milliliter, was used for each replication, resulting in a total of eight petri dishes. Immature ovaries were added to the culture at a density of one per milliliter of medium immediately preceding the incubation. Ovaries were aseptically picked out from fresh and disinfected spikes of genotype Chris. The petri dishes were sealed with Parafilm and incubated in the dark at 27°C.

When embryos reached the size of 1–2 mm, they were transferred aseptically to solid 190-2 medium (Zhuang and Xu 1983) at a density of 20 embryos in each 100×15 mm petri dish. They were incubated at room temperature (22°±3 C) with 150–180 µmol $m^{-2}$ $s^{-1}$ of illumination provided by continuous fluorescent light. Within approximately 2 weeks, green plants developed and were ready for transfer to soil. Green plants were raised in the greenhouse, much like plants grown from seeds.

### Data analysis

The experiment was considered as a completely randomized design. There were two treatments, each replicated twice. Two genotypes were used. Each petri dish containing microspores from the three spikes of the same treatment was considered as one replication. The general linear model (Lentner and Bishop 1993) was used to statistically analyze the data. Analysis of variance was conducted, followed by the 5% least square difference analysis, for five traits – i.e. number of embryos, regeneration rate, green versus albino plant percentage and spontaneously DH percentage. At 45 days after microspore plating, embryos visible to naked eyes were counted, while smaller structures were ignored. We used only a small portion of the embryos that matured in the early phase for evaluating embryo quality. To avoid bias, we transferred the first available 115 and 95 embryos from each petri dish to evaluate plant regeneration rate for the genotypes WED 202-16-2 and Svilena and DH percentage for WED 202-16-2, respectively. Embryos were transferred to regeneration media in three groups based on the size of embryos between 30 days and 45 days after microspore plating. Green and albino plants with well-developed roots and shoots were counted at 14 days after embryos had been transferred to regeneration media. Between 13 and 20 plants for each treatment were evaluated for ploidy level based on fertility (10 or more seeds per spike). The experiment was repeated more than three times. The same treatment was also applied to some NPB breeding lines for DH production.

## Results and discussion

### Embryo induction

All petri dishes produced a similar number of embryos at 45 days after microspore plating (Table 1, Fig. 1a). Embryos eventually completely covered the entire bottom of all petri dishes. When larger embryos were removed and the culture media and ovaries were refreshed weekly, hundreds more multi-cellular structures or pre-embryos developed into mature embryos. Each petri dish continuously produced more than 1,000 embryos over a period of several months. Thus, the presence of additional nutrients in the pretreatment solution during the initiation of microspore embryogenesis did not affect embryo production. This indicates that when microspores are being switched from the programmed gametophytic towards the sporophytic pathway by a chemical inducer formulation under proper physiological conditions, additional nutrients available to microspores are not the essential factors for inducing the formation of embryos. Nutrients

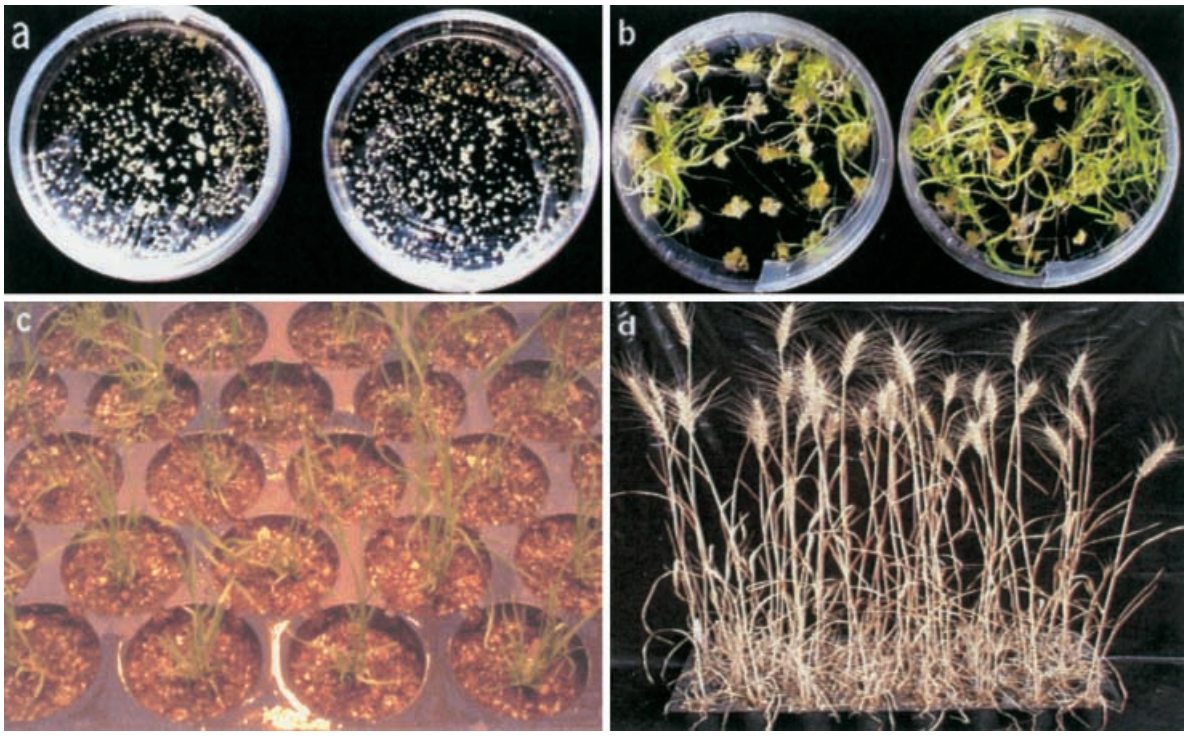


**Fig. 1a–d** Embryo formation, green plant regeneration and DH production as affected by nutrients during pretreatment, genotype WED 202-16-2. **a** Embryo formation at 45 days after microspore plating: control (*left*) and with nutrients (*right*). **b** Plant regeneration at 14 days on regeneration medium: control (*left*) and with nutrients (*right*). **c** Microspore-derived plants 1 week after transfer to greenhouse. **d** Microspore-derived DH plants

**Table 1** The effect of nutrients in the pretreatment solution on plant regeneration from microspores

| | WED 202-16-2[a] | | | Svilena[a] | | |
|---|---|---|---|---|---|---|
| | Nutrient | Control | LSD(0.05) | Nutrient | Control | LSD(0.05) |
| Number of visible embryos at day 45 | 620A | 640A | | 624A | 650A | |
| Plant regeneration[b] (%) | 70A | 61B | 4 | 92A | 88B | 3 |
| Green plant regenerants[c] (%) | 89A | 70B | 4 | 95A | 94A | 7 |
| Albino plant regenerants[c] (%) | 11A | 30B | 4 | 5A | 6A | 7 |
| DH plants[d] (%) | 63A | 64A | 52 | | | |

[a] Means followed by the same letter in the same row are not significantly different by ANOVA and 5% LSD analysis
[b] Plant regeneration (%)=100 × (no. of green and albino plants)/no. of embryos plated
[c] Green or albino plant regenerants (%)=100 × (no. of green or albino plants)/no. of regenerants
[d] DH plants (%)=100 × (no. of DH plants/no. of green plants evaluated)

present in tissues such as anther walls and stem tissue surrounding microspores may be the nutrient source during the pretreatment. Microspores are not really completely "starved" in this pretreatment regime.

## Plant regeneration

For the genotype WED 202-16-2, the addition of 10% NPB98 culture medium in the pretreatment solution significantly increased both the plant regeneration rate (15% increase) and the percentage of green plants (27% increase) among regenerants ($P$=0.05) (Table 1, Fig. 1b). This result shows that, with respect to a "problematic" genotype, such as WED 202-16-2, the availability of additional nutrients to the microspores during embryo initiation helped enable a large population of microspores to develop into embryos of good quality that were competent for regenerating into green plants, while it did not negatively affect the growth of embryos from a genotype with a "good" genetic background, such as Svilena (Table 1).

The regenerated plants from each treatment looked similar, and no significant difference in the spontaneous DH frequency was detected for the genotype WED 202-16-2 (Table 1, Fig. 1c, d). Svilena plants were not evaluated for the percentage of spontaneous DHs because there was no difference in green plant regenerants.

Similar results for genotypes WED 202-16-2 and Svilena were obtained when the experiments were repeated. When the same nutrient treatment was applied to some NPB breeding lines, there was a significant improvement in green plant frequency among regenerants in those genotypes having a moderate to high frequency of albinos (data not shown).

Our experiments demonstrated that an increase in green plant production for "problematic genotypes" can be achieved by means of a simple alteration to the established pretreatment conditions – i.e. the addition of nutrients to the pretreatment solution in the flask. Thus, it is possible to maintain the high efficiency of embryo production while increasing the proportion of green plants regenerated. The overall efficiency, as measured by total green plant production per spike, was high. More than 100 green plants per spike could be produced for genotype WED 202-16-2 (Table 1). Thus, the current isolated microspore culture system is efficient for haploid/doubled haploid production in wheat. Other reports indicate that the ratio of green to albino plants could be affected by several factors, including the temperature during pretreatment for inducing microspore embryogenesis (Hu and Kasha 1999). Previous experiments at NPB have shown that lower temperature stress (4–30°C) during the pretreatment increases the proportion of green plants formed. However, this increase in the ratio of green to albino plants by the use of a lower stress temperature caused a significant reduction in the number of embryos. As a result, total green plant production per spike was sharply reduced (data not shown). Thus, a better strategy was to maintain the high efficiency for embryo production and to improve embryo quality, i.e. competency for a higher frequency of green plant regenerants.

This experiment sheds some light on the albino propensity of certain genotypes. We observed that androgenesis is influenced by the genetic background of the mother of particular genotypes. However, environment is an important factor affecting plant regeneration and green plant percentage (Zhou and Konzak 1997). We were able to demonstrate that an apparently genetic influence on androgenesis can be overcome, to some extent, by providing improved conditions to microspores induced to androgenesis. There are reports that albino plants in wheat and barley have altered plastids in which the DNA has been changed or partially deleted (Day and Ellis 1984, 1985; Chen et al. 1986). We show here that nutrients available to microspores during pretreatment at the beginning of embryogenesis were decisive in determining the quality of embryos with respect to regeneration ability and green plant propensity. The environment surrounding the microspores drastically changes when spikes containing microspores are cut and subjected to a stress treatment in vitro. The question still remains as to how the lack of nutrients readily available to microspores relates to alterations or deletions in plastid DNA. Whatever is behind the process, the immediate availability of additional nutrients to microspores during the in vitro pretreatment seems to be critical for the formation of good-quality embryos with a higher percentage of green regenerants.

In conclusion, our results clearly show that making some nutrients available to microspores at the stage when embryogenesis is triggered is an important factor affecting plant regeneration and green plant production in the NPB flask culture system for a genotype with a relatively high propensity for albinism. While stresses including reduced nutrient availability may be beneficial for the induction of androgenesis, some nutrients are needed for normal green plant formation at the very beginning of androgenesis induction. This is especially true for genotypes that frequently produce albino plants. Furthermore, the presence of additional nutrients during pretreatment does not seem to inhibit a high efficiency of embryo production with responsive genotypes. Thus, high-efficiency green plant production per unit of microspores can be obtained. Further study should be directed towards determining if the observed responses are due to major or minor nutrients, or maltose, and towards determining the effects of nutrient concentration and treatment duration.

## References


Anderson SB, Due IK, Olesen A (1987) The response of anther culture in a genetically wide material of winter wheat (*Triticum aestivum* L.). Plant Breed 99:181–186

Chen YR, Huang YF, Lin TL (1986) Variations of plastids in pollen-derived albino plants of rice. In: Bailey GW (ed) Proc 4th Annu Meet Electron Microsc Soc Am, vol 44. San Francisco Press, San Francisco, pp 196–197

Day A, Ellis THN (1984) Chloroplast DNA deletions associated with wheat plants regenerated from pollen: possible basis for maternal inheritance of chloroplasts. Cell 39:359–368

Day A, Ellis THN (1985) Deleted forms of plastid DNA in albino plants from cereal anther culture. Curr Genet 9:671–678

Hu H (1997) In vitro induced haploids in wheat. In: Jain MS, Sopory SK, Veilleux RE (eds) In vitro haploid production in higher plants. Kluwer, Dordrecht, pp 73–97

Hu H, Yang HY (1986) Haploids of higher plants in vitro. China Academic/Springer, Beijing/Berlin Heidelberg New York

Hu T, Kasha KJ (1999) A cytological study of pretreatments used to improve isolated microspore cultures of wheat (*Triticum aestivum* L.) cv. Chris. Genome 42:432–441

Konzak CF, Sunderman DW, Polle EA, McCuiston WL (1987) Spring wheat plant design for conservation tillage crop management systems. In: Elliott L (ed) STEEP – conservation concepts and accomplishment. Washington State University Publications, Pullman, pp 247–273

Lentner M, Bishop T (1993) Experimental design and analysis. Valley Book, Blacksburg, Va.

Liu W, Zheng YM, Polle E, Konzak CF (2001) Highly efficient doubled-haploid production in wheat (*Triticum aestivum* L.) via induced microspore embryogenesis. Crop Sci (in press)

Tuvesson IKD, Perdersen S, Andersen SB (1989) Nuclear genes affecting albinism in wheat (*Triticum aestivum* L.). Theor Appl Genet 78:879–883

Zhou H, Konzak CF (1997) Influence of genetic and environmental factors on anther culture response of wheat. J Appl Genet 38:393–406

Zhuang JJ, Xu J (1983) Increasing differentiation frequencies in wheat pollen callus. In: Hu H, Vega MR (eds) Cell and tissue culture techniques for cereal crop improvement. Science Press, Beijing, p 431