

# AN EFFICIENT HIGH THROUGHPUT BRASSICA NAPUS MICROSPORE CULTURE SYSTEM: INFLUENCE OF PERCOLL GRADIENT SEPARATION AND PRECISE BUD SELECTION ON EMBRYOGENESIS

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## Introduction

Microspore culture for the purpose of developing double haploid plants is routine for numerous plant species; however, the embryo yield is still very low compared with the total microspore population (Pechan and Keller, 1988). The ability to select and isolate highly embryogenic microspores would be of great advantage for high embryo yield in microspore culture. To maximize the embryogenic frequency in canola microspore culture, we followed a combination of precise bud size selection and microspore fractionation using a percoll gradient. In addition, we studied the relationship between bud size, developmental stage of isolated microspores, percoll gradient concentration and the embryogenic frequency of each cultivar.

## Materials and Methods

**Plant Material:** *Brassica napus* cvs. Topas 4079, DH12075, Westar, and 0025 were grown in growth cabinets with a 16 hour photoperiod at 20°C (day) and 15°C (night). Prior to bolting, the chamber temperature was decreased to 10°C (day) and 5°C (night).

**Microspore Culture:** As described in Ferrie (2003), fifty buds per bud size range (Figure 1) were surface sterilized and the microspores were released by crushing the buds in 1/2 strength B5-13% sucrose wash media. The microspore suspension was centrifuged at 150 g for 3 min. The pellet was washed two more times and an aliquot of microspores was counted on a hemacytometer to determine density. The microspores were resuspended in NLN-13% sucrose, 0.08% glutamine (NLN-13) at  $5 \times 10^4$  microspores per mL and cultured at 32°C for 3 days then 24°C continuous. Embryos were counted 21 days after microspore extraction.

**Percoll Density Gradient Centrifugation:** Microspores obtained from 50 buds of 2.0-2.5 mm DH12075 were extracted as above, resuspended in NLN-13, and carefully transferred onto a 10, 20, and 40% percoll gradient. The microspores were spun at 100 g for 5 min and individual populations were removed from the resulting bands. The microspores were washed and plated as above.

## References

Ferrie, A. M. R. (2003) Microspore culture of *Brassica* species. Doubled haploid production in crop plants 205-215. Maluszynski M. Kasha K. J. Forster B. P. Szarejko I. Dordrecht. Kluwer Academic Publishers.

Pechan, P.M. and Keller, W. (1988) Identification of potentially embryogenic microspores in *Brassica napus*. *Physiol. Plant.* 74:377-384

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## Results and Discussion

Microspores isolated from buds of Topas 4079, DH12075, Westar and 0025 in the range of 1.5 to 4.5 mm formed embryos at various frequencies (see Figure 2). Although both Topas 4079 and DH12075 are very embryogenic cultivars, Topas 4079 exhibited a larger window of embryogenic bud sizes than DH12075. Westar is a less embryogenic cultivar but produced embryos over a wide range of bud sizes. *B.n.* 0025 has been reported as non-embryogenic (Simmonds and Keller, 1999) although responded well at the specific bud size of 2.5-3.0 mm. Day 21 embryo counts of three replicate experiments were used to obtain average numbers of embryos per 100 buds (Figure 3). Although embryos were obtained from many bud size ranges, the most embryogenic bud size range varied with each cultivar: Topas 4079 3.5-4.0 mm, DH12075 2.0-2.5 mm, and Westar and 0025 2.5-3 mm. The quality of the embryos obtained from smaller buds was consistently better than embryos obtained from larger buds. For example, 3.0-3.5 mm buds of Topas 4079 consistently yielded further developed embryos by Day 21 than the larger bud sizes.

As an individual bud may contain microspores of various developmental stages, different microspores found within a single bud size range were further separated by percoll density gradient centrifugation. When the microspores from 2.0-2.5 mm buds of DH12075 were carefully layered on top of a percoll gradient and spun through the percoll layers by centrifugation, bands were formed containing microspores of different developmental stages. The concentrations of percoll used in the gradient were optimized for the microspores obtained from a specific bud size. The centrifugation speed and time was optimized as well. Figure 4 displays microspore populations recovered from different bands of the percoll gradient. Generally, the early and mid uninucleate microspores separated in the top layer and the dense, late uninucleate and binucleate microspores separated in the middle layer. Staining with DAPI (Figure 4 insert) confirmed the nuclear stage of the recovered microspores.

To ensure that the percoll separation step was not detrimental to microspore embryogenesis, the recovered microspores were washed and plated for culture. Figure 5 compares the embryos obtained in control plates with those microspores recovered from a separate band of the percoll gradient. The percoll embryos were more uniform as the microspores obtained were at approximately the same stage of development.

These approaches have repeatedly given us enhanced embryo yields. The ability to select optimum bud size and potentially embryogenic microspore populations would facilitate in depth studies of embryo induction and development in many other crop species.

Figure 1. Buds separated according to bud size ranges

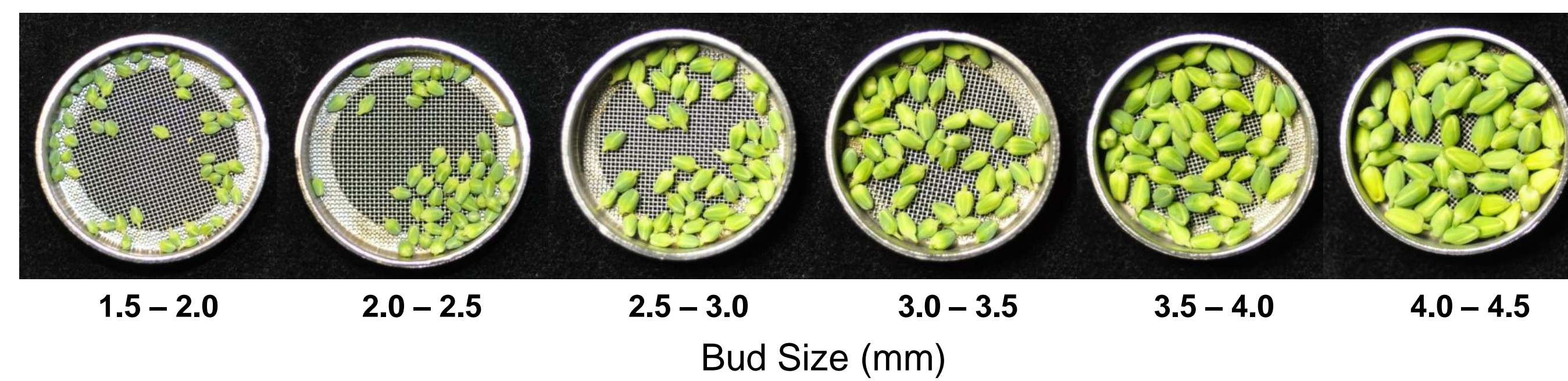


Figure 2. Embryos resulting from microspore culture of different bud size ranges

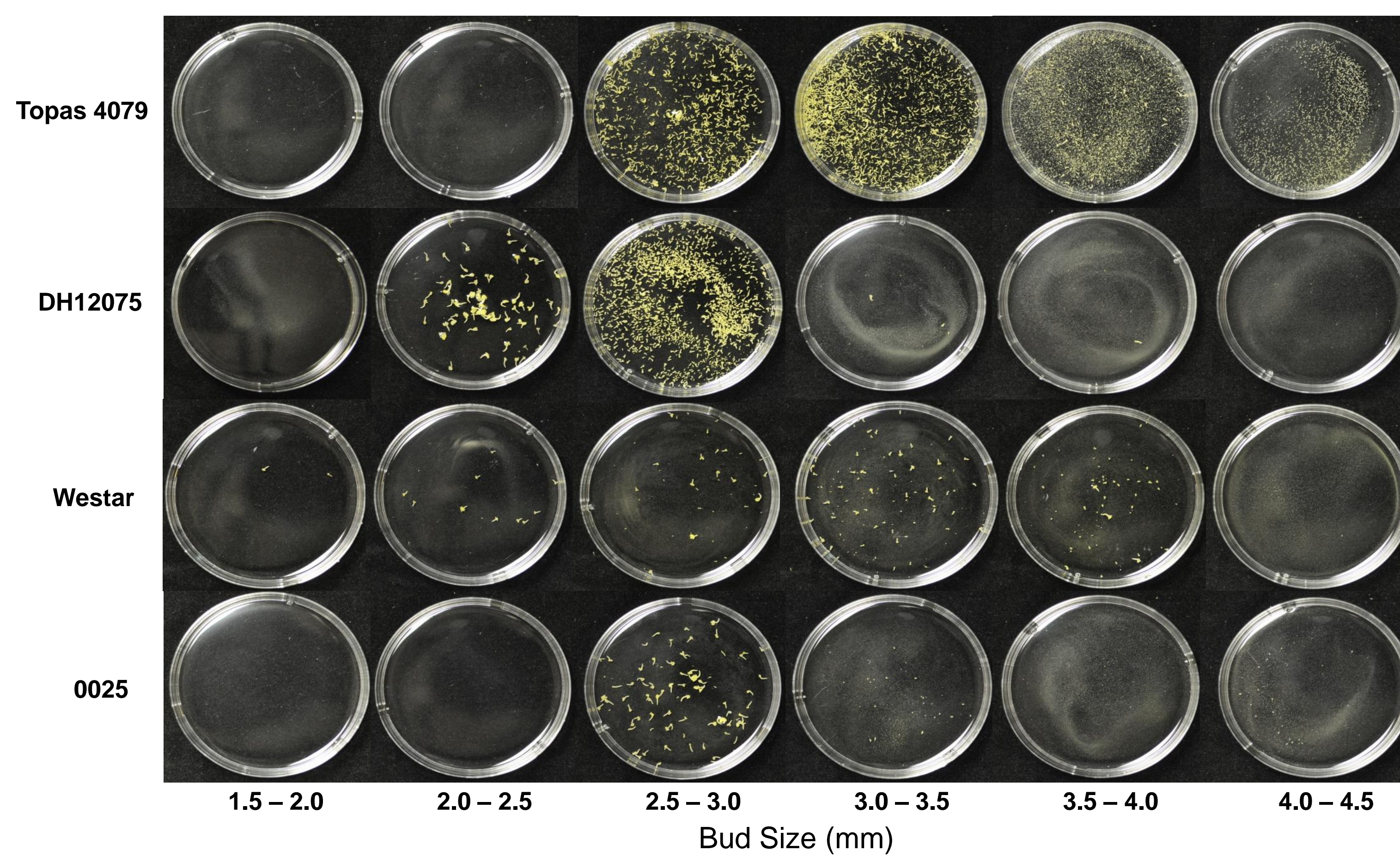


Figure 3. Embryos resulting from microspore culture of different bud size ranges

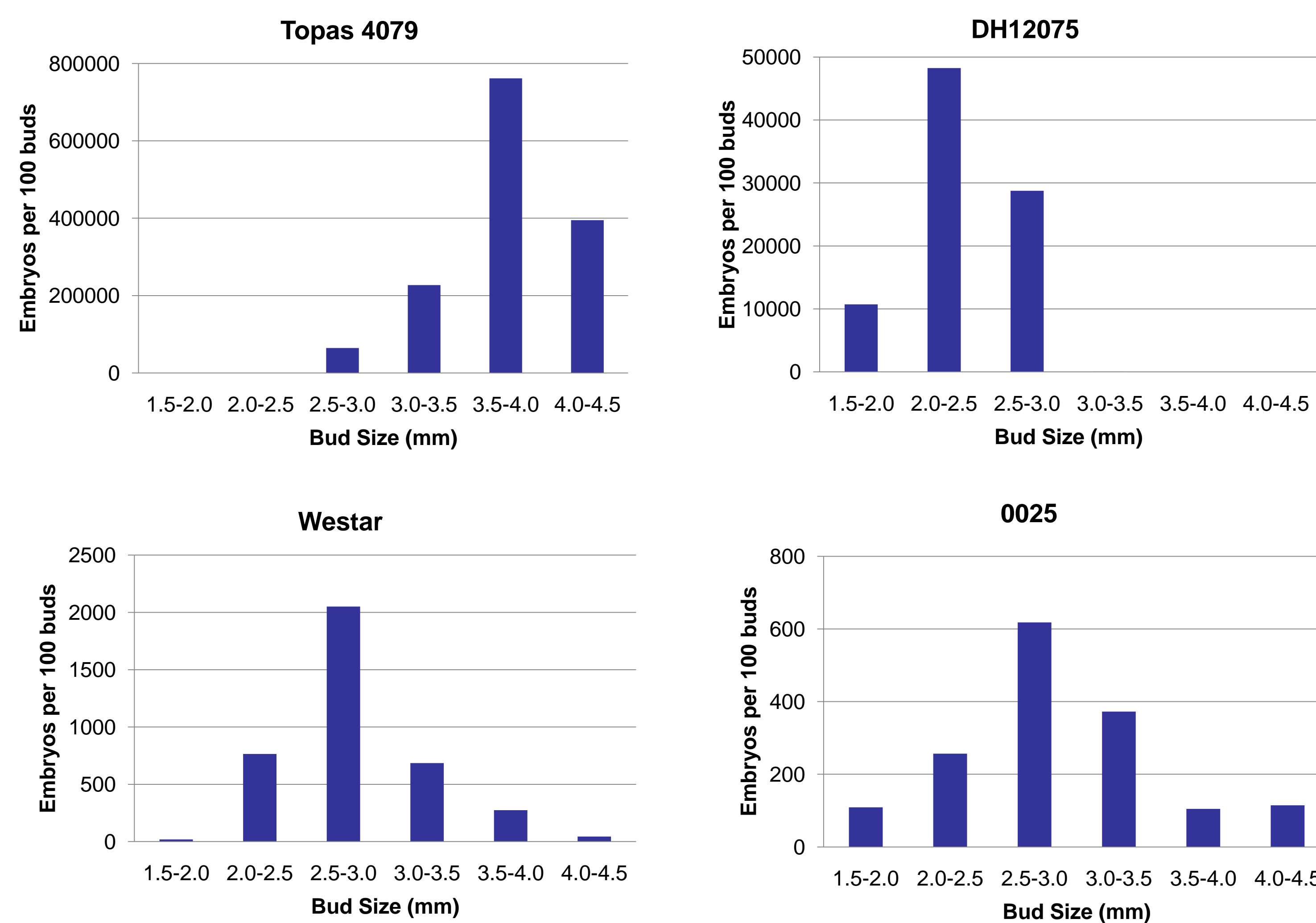


Figure 4. DH12075 microspores recovered from top and middle layers of percoll gradient.

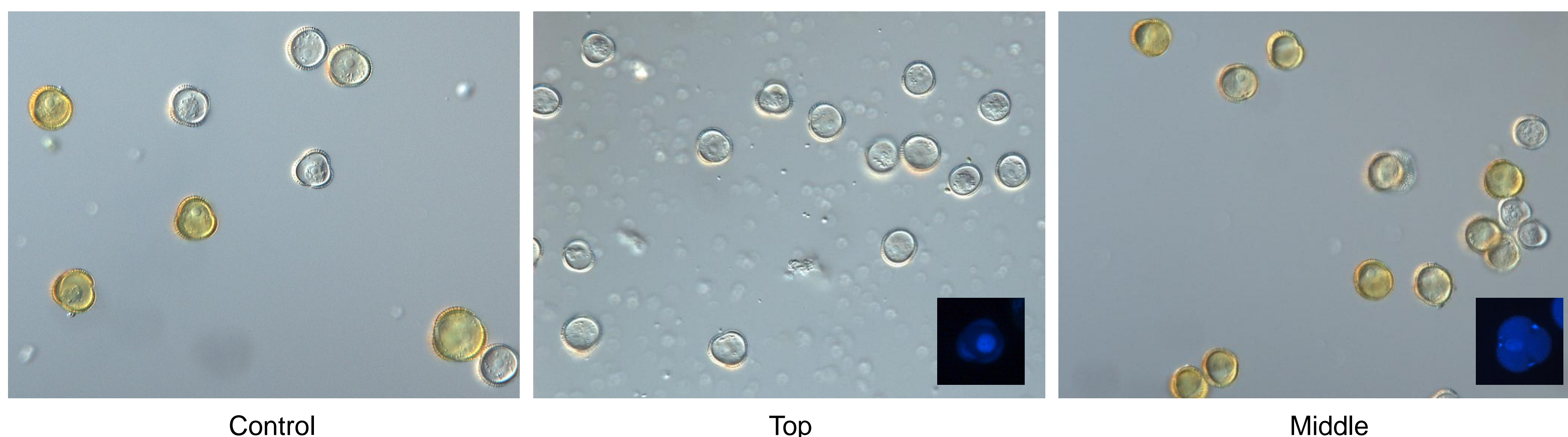


Figure 5. DH12075 embryos from microspores separated using percoll

