Cryopreservation of isolated microspores of spring rapeseed (*Brassica napus* L.) for *in vitro* embryo production

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**Abstract**

Microspore cryopreservation is a potentially powerful method for long-term storage of germplasm for *in vitro* embryo production in plant species. In this study, several factors influencing embryo production following the ultra-low temperature (−196°C in liquid nitrogen) storage of isolated microspores of rapeseed (*Brassica napus* L.) were investigated. Microspores were prepared in cryogenic vials and subjected to various cooling treatments before immersion in liquid nitrogen for varying periods. Efficiency of microspore cryopreservation was reflected by *in vitro* embryo production from frozen microspores. Of all the cooling treatments, microspores treated with a cooling rate of 0.25% °C/min and a cooling terminal temperature of −35 °C before immersion in liquid nitrogen produced the highest embryo yields (18% and 40% of unfrozen controls in two genotypes, respectively). Fast thawing in a 35 °C water bath was necessary to recover a high number of embryos from microspore samples being frozen at a higher cooling rate, while thawing speed did not affect samples after freezing at a slower cooling rate. The storage density of cryopreserved microspores affected embryo production. Storage at the normal culture density (8 × 10⁴ microspores/ml) was less efficient for embryo production than at high densities (4 × 10⁶ microspores/ml and 1.6 × 10⁷ microspores/ml), although no significant difference was found between the high densities. Evaluation of plant lines derived from frozen microspores indicated no variation in isozyme pattern and no enhanced cold tolerance of these lines. Isolated microspores of *B. napus* could be stored for extended period for *in vitro* embryo production.

**Introduction**

Cryopreservation of biological materials is simply the preservation of those material in a frozen state. Technically, cryopreservation usually implies storage at very low temperatures: in the vapour phase over liquid nitrogen (−150°C) or more commonly in liquid nitrogen itself (−196°C). The process of cryopreservation can slow considerably or even halt cellular metabolism and biological deterioration, since at under such a low temperature, kinetic energy levels are too low to allow the necessary molecular motion required for normal cellular activities (Grout et al. 1990). Cryopreserved material remains genetically stable and the genetic drift which is a phenomenon of organisms maintained by standard techniques can be avoided.

The development of the isolated microspore culture system of *Brassica napus* L. (Chuong & Beversdorf 1985; Lichter 1982; Polsoni et al. 1988; Swanson et al. 1987) has potential for application in haploid breeding programs (Charne 1990; Chen & Beversdorf 1990a, b). The provision of isolated microspores, therefore, may become a fundamental requirement in plant biology and breeding. Although genetic stocks currently are preserved in seed collections, mi-
Microspore preservation serves as a supplementary approach to seed storage, especially with the advent of biotechnology. By employing microspore preservation, efficient use of resources is promoted: germplasm can be preserved in large quantities and minimum space and simple facilities are required. Storage of microspores would facilitate an even distribution of labour, time and space for microspore culture to directly produce homozygous lines of each genotype. Physiological and biochemical studies also require microspores to be stored in a constant condition over time.

Low temperature preservation is very effective not only for maintaining germplasm for conventional breeding, e.g. oilseed crops (Bajaj 1990), but also for the storage of mature pollen, vegetative stems, and cell and protoplast suspensions of many species (Kartha 1985a). Bajaj (1983) made an early attempt to cryopreserve the pollen-embryos and segments of the androgenic anthers of *B. napus* for one year. However, little research has been reported regarding the cryopreservation of isolated microspores of rapeseed (*B. napus*), although the preliminary attempt to cryopreserve microspores was made by Charne et al. (1988) with limited success. The objectives of the current study were to investigate several factors influencing *in vitro* embryo production following the cryopreservation of isolated microspores of rapeseed (*B. napus*).

Materials and methods

**Microspore donor and microspore extraction**

Two homozygous spring rapeseed (*B. napus*) breeding lines, G-231 (a moderately embryogenic line) and M3-124 (a highly embryogenic line), were used as microspore donors. Ten plants from each line grown to flowering in a growth room provided microspores for freezing studies. A mixture of cool white and Gro-lux wide spectrum fluorescent lights provided a photosynthetic photon flux density of 270 μmol m⁻² s⁻¹ during a 16-h/24 photoperiod with a 23/19°C day/night temperature regime. Young flower buds shorter than 4.5 mm in length were collected in bulk for each genotype. The microspores were extracted based on the method described by Polsoni et al. (1988). Buds were surface sterilized and ground in B₅ liquid medium with a blender. After centrifuging, the microspore pellets were washed three time in B₅ liquid medium.

**Cooling, cryopreservation and thawing**

**Effect of cooling rate and cooling terminal temperature**

Isolated microspores of G-231 and M3-124 were resuspended in pre-chilled (4°C) Lichter’s media (Lichter 1982) supplemented with 13% sucrose as both a nutrient component and a cryoprotectant at a density of 4 × 10⁶ microspores/ml. A 3 × 4 factorial experiment was designed for each genotype. The initial cooling temperature was 5°C. Cooling rate (cold treatment before quenching in liquid nitrogen), R, was one factor with three levels: 0.03°C/min, 0.25°C/rain and 1°C/min. The cooling terminal temperature (temperature before immersion in liquid nitrogen), T, was the second factor with four levels: -25°C, -30°C, -35°C and -40°C. Treatments were arranged in a randomized complete block design (RCBD) with three replications. Each treatment utilized a 1 ml Corning cryogenic vial (Fisher Scientific, Mississauga, Canada) containing 1 ml of microspore suspension. Once terminal temperature was reached following the various cooling rates, the vials were plunged immediately into liquid nitrogen and stored for 3 days before being thawed in a 35°C water bath.

Cooling was facilitated by the use of a computer-programmable methanol bath, Hakke H Model B3 (Fisher Scientific, Mississauga, Canada).

**Effects of cooling rate and thawing procedure**

A 2 × 2 factorial design was used to determine the effects of cooling rate and thawing procedure. The initial cooling temperature was 5°C. The cooling rate was set at two rates: low (0.03°C/min) and high (1°C/min), while the thawing procedure included two levels: 35°C water bath and air at room temperature (22°C) until melting ice was complete. The four treatments were arranged as a RCBD with three replications. Each treatment utilized 1 ml of mi-