A novel approach to microspore embryogenesis in *Brassica napus* L.

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By

Katica Ilić-Grubor

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SUMMARY OF DISSERTATION

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by

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A NOVEL APPROACH TO MICROSPORE EMBRYOGENESIS IN Brassica napus L.

The objective of this study was to investigate development of haploid embryos of *Brassica napus* induced from isolated microspores, and to provide a comparison to the corresponding developmental stages of zygotic embryos. A novel method for induction and culture of microspore-derived (MD) embryos was designed, based on restricted sucrose supply and use of high molecular weight polyethylene glycol (PEG) as an osmoticum. Haploid embryos cultured under these conditions were studied, from the induction phase to the formation of cotyledonary embryos, through their maturation and desiccation. Finally, plantlet formation and their *ex vitro* acclimatization were assessed.

Induction and formation of MD embryos was not affected by limited sucrose supply; embryos proceeded through globular, heart, torpedo and cotyledonary stages within two weeks. After exposure to light, PEG embryos turned dark green and appeared similar to dissected early cotyledonary stage zygotic embryos.

Morphological changes during the time-course of microspore embryogenesis were studied using scanning electron microscopy. Early embryo development from embryogenic microspores to the globular stage was irregular and differed from that of zygotic embryos. However, at heart, torpedo and cotyledonary stages, PEG embryos were remarkably similar to their zygotic counterparts in size and shape, with well-developed cotyledons. Sucrose embryos were 2-3 times larger than PEG embryos, but cotyledons were small and poorly differentiated. Numerous large starch grains were observed in cells of sucrose embryos at the early cotyledonary stage, but were almost completely absent in PEG embryos. The characteristic presence of pollen wall remnants suggested an origin of polarity in MD embryos, possibly established in late uninuclear microspores and early bicellular pollen.

Application of ABA and desiccation treatment improved the *in vitro* conversion frequency of both sucrose and PEG embryos. However, PEG plantlets directly transferred to soil had a higher survival rate and enhanced vigor during the acclimatization *ex vitro*.

This novel microspore culture system is suitable for studies of *in vitro* embryogenesis with a broad range of plant species and should have important potential application in plant biotechnology and breeding programs.

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CERTIFICATION OF THESIS WORK

We, the undersigned, certify that **Katica ILIC-GRUBOR** Doctor of Philosophy has presented a thesis with the following title: ***"A Novel Approach To Microspore Embryogenesis In Brassica napus L"***. We consider that the thesis is acceptable in form and content, and that the candidate through an oral examination held on August 24, 1998 demonstrated a satisfactory knowledge of the field covered by the thesis.

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ABSTRACT

The objective of this study was to investigate in detail development of haploid embryos of *Brassica napus* L. induced from isolated microspores, and to provide a comparison to the corresponding stages of zygotic embryo development *in vivo*. A novel method for induction and culture of microspore-derived (MD) embryos was designed, based on restricted carbohydrate supply and use of high molecular weight polyethylene glycol (PEG 4000) as an osmoticum. Haploid embryos induced and cultured under these conditions were studied throughout their development, from the induction phase to the formation of cotyledonary embryos, through maturation and desiccation. In the final phase, plantlet formation and *ex vitro* acclimatization were assessed.

Induction and formation of MD embryos in PEG medium was not affected by limited sucrose supply; embryos proceeded through globular, heart, torpedo and cotyledonary stages within two weeks. After exposure to light, MD embryos quickly turned dark green and appeared similar to dissected early cotyledonary stage zygotic embryos. The conversion frequency of PEG embryos and sucrose embryos (control) was 30-50%, with the highest values achieved with four-week-old MD embryos.

Morphological changes during the time-course of microspore embryogenesis were studied using scanning electron microscopy. Early embryo development from embryogenic microspores was irregular and differed from that of zygotic embryos which exhibited a precise cell division pattern. However, at the heart, torpedo and cotyledonary stages, PEG embryos were remarkably similar to their zygotic counterparts in size and shape, with well-developed cotyledons. Sucrose embryos were 2-3 times larger than PEG embryos, but cotyledons were small and poorly differentiated. Numerous large starch grains were observed in cells of sucrose embryos at the early cotyledonary stage, but were almost completely absent in PEG

embryos. The characteristic presence of pollen wall remnants suggested an origin of polarity in MD embryos, possibly established in late uninuclear microspores and early bicellular pollen.

Exogenous ABA and desiccation treatment improved the *in vitro* conversion frequency of both sucrose and PEG embryos. However, PEG plantlets directly transferred to soil had a higher survival rate and enhanced vigor during the acclimatization *ex vitro*. Direct transfer to soil offered several advantages over traditional *in vitro* plantlet conversion on solid medium, since normal vigorous plantlets were obtained in a greenhouse within ten to twelve weeks.

This novel microspore culture system is suitable for studies of *in vitro* embryogenesis with a broad range of plant species and should have important potential application in biotechnology and breeding programs.

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LIST OF ABBREVIATIONS

ABA	=	abscisic acid
°C	=	degree Celsius
CPD	=	critical point drying
dH_2O	=	distilled water
DAPI	=	4′-6-Diamidino-2-phenylindole · 2HCl
DPA	=	days post anthesis
DW	=	dry weight
Fig	=	Figure
FW	=	fresh weight
g	=	gram
h	=	hour
HXK	=	hexokinase
L	=	liter
LEA	=	Late Embryogenesis Abundant
MD	=	microspore-derived
mOs/kg H_2O	=	milliosmoles/kilogram of water
MPa	=	megapascal
NLN	=	Lichter's medium (1982)
µg	=	microgram
µL	=	microliter
µM	=	micromolar
µm	=	micrometer
µmoles/g	=	micromoles/gram
PAS	=	Periodic acid-Schiff's reaction
PEG	=	polyethylene glycol
SEM	=	scanning electron microscopy
ψ_s	=	osmotic potential

Section 1. GENERAL INTRODUCTION

1.1. Importance of oilseed *Brassica* species

The oilseed *Brassica* species (*B. napus*, *B. rapa* and *B. juncea*) constitute the third most important source of vegetable oil worldwide (Shahidi, 1990). Commercial oilseed varieties of *B. napus* and *B. rapa* known as canola have low erucic acid and low glucosinolate levels. The term "canola" is, in fact, a trademark registered with the Canola Council of Canada and applied to varieties with 2% or less erucic acid and less than 30 μmoles/g glucosinolates in oil free meal (Canola Growers Manual, 1990). The first variety of canola, var. Tower (*B. napus*), was registered in 1974 in Manitoba (Stefansson and Kondra, 1975). Several others known as "double low" varieties of canola were registered shortly after, marking a milestone in oilseed production in Canada. As a result, over the last two decades, canola has become the most important oil crop in Canada and Europe.

With over 40% oil content, canola seed has been used mainly for oil production. It has also been used as a high protein animal feed, since seed contains 20-25% protein (Finlayson, 1976). However, the presence of glucosinolates in the meal after oil extraction reduces its nutritional quality, despite the high protein levels. Further genetic improvement of canola and related *Brassica* oilseed species has been a constant goal for canola breeders whose efforts are directed towards:

i.) development of new varieties with an increased oil content and modified seed oil composition (high oleic acid and low linoleic acid canola varieties),

ii.) improved seed and meal quality (reduction of glucosinolates and phytic acid),

iii.) increased pest and disease resistance,

iv.) selective herbicide resistance,

v.) enhanced tolerance to environmental stresses.

Realization of these complex breeding programs within a shortened time frame is now feasible due to the combination of conventional breeding tools with modern biotechnological tools, such as genetic engineering and microspore culture.

1.2. The discovery of *in vitro* haploid embryogenesis from anthers and pollen grains

Normal development of the male gametophyte in angiosperms follows a programmed sequence of events commencing with meiosis of the pollen mother cell and terminating with the formation of mature bicellular or tricellular pollen grains. The discovery that pollen grains can switch from their gametophytic developmental mode to an embryogenic pathway and develop into haploid embryos resulted from experiments on isolated anther culture of *Datura* (Guha and Maheshwari, 1964, 1966). This partly accidental discovery (Maheshwari, 1996) triggered a massive investigation of the phenomenon of haploid embryo induction from male gametophytes *in vitro*. Shortly after the success in *Datura*, haploid embryogenesis through anther culture was reproduced in tobacco (Bourgin and Nitsch, 1967) and rice (Niizeki and Oono, 1968), followed by the first success in haploid embryo induction from isolated microspores in tobacco (Nitsch and Norreel, 1974). Since then, *in vitro* haploid embryogenesis has been demonstrated through anther and/or isolated pollen culture in over 200 species of flowering plants (see Srivastava and Johri, 1988). The embryogenic developmental pathway in which haploid embryos develop from the male gametophyte *in vitro* is usually referred to as microspore embryogenesis, regardless of whether the embryos originate from unicellular microspores or bicellular pollen (Touraev et al., 1997).

The realization that microspore embryogenesis can be induced in a large number of flowering plants changed our understanding of developmental processes in plants and raised a series of fundamental questions. How is the natural course of microsporogenesis altered to the

embryonic pathway and what precise mechanism triggers this switch? Why do genotype responses for haploid induction vary so profoundly even within species? How is microspore and pollen competence for haploid induction achieved at specific developmental stages? Thirty five years after the discovery of this phenomenon, the answers to these and many other questions are still largely unknown.

1.3. The importance of double haploids

The value of haploids in genetic research and in plant breeding has been recognized for a long time. Since the first natural haploid plant was discovered in *Datura stramonium* (Blakeslee et al., 1922), the search for these haploids, derived from male and female gametophytes, resulted in reports on naturally occurring haploids in about 100 species of flowering plants (Vasil, 1996). However, they occur rarely in nature, and use of these natural haploids was extremely limited. Therefore, several strategies were identified for the production of haploids (see Ferrie et al., 1994; Kush and Virmani, 1996). After the first success in induction of haploid embryogenesis via anther culture over three decades ago, induced haploidy from the male gametophyte suddenly emerged as one of the most attractive routes for production of large numbers of haploid plants.

The advantages of using double haploids over conventional breeding methods are:

i.) shortened breeding programs in developing new varieties (single step production of homozygous double haploid lines),

ii.) screening of the recessive traits (genotype masking by dominant genes does not occur in double haploids),

iii.) production of homozygous double haploid lines (inbred lines) in self incompatible species, such as *B. rapa*.

The high frequency of haploid embryos obtained using microspore culture has made it a

suitable and important tool in selection in *Brassica* breeding programs (reviewed by Palmer et al., 1996), e.g., mutagenesis and selection (Polsoni et al., 1988; Swanson et al., 1989; reviewed by Kott et al., 1996) and genetic transformations (reviewed by Huang, 1992; Poulsen, 1996).

1.4. Progress in *Brassica* anther and pollen culture

In *Brassica napus*, induction of haploid embryos *in vitro* progressed from a modest success in immature anther culture (Thomas and Wenzel, 1975; Keller and Armstrong, 1977), through the first embryo induction from isolated microspores (Lichter, 1982), towards development of a highly efficient haploid embryogenic system (Chuong and Beversdorf, 1985; Pechan and Keller, 1988; Telmer et al., 1992). Such an efficient haploid system was the result of investigations of a number of important factors which affect microspore embryogenesis in *Brassica napus*:

i.) donor plant growth conditions (Keller et al., 1987; Chuong et al., 1988; Takahata et al., 1991),

ii.) genotype variation (Chuong and Beversdorf, 1985; Chuong et al., 1988; Gland et al., 1988),

iii.) culture medium composition (Lichter, 1982; Gland et al., 1988),

iv.) developmental stage of gametophytic cells (Fan et al., 1988; Pechan and Keller 1988; Zaki and Dickinson 1991; Telmer et al., 1992; Binarova et al., 1997),

v.) pretreatments (heat shock treatment - Keller and Armstrong, 1978; ethanol and gamma irradiation - Pechan and Keller, 1989; embryo induction with colchicine - Mathias and Robbelen, 1991; Zhao et al., 1996),

vi.) culture conditions (Chuong and Beversdorf, 1985; Swanson et al., 1987; Huang et al., 1990).

All of these factors are important for the regular production of high numbers of microspore-derived (MD) embryos. Among the studies listed above, the major landmarks in developing

the microspore culture system in *B. napus* included, first of all, the initial success in culturing isolated microspores (Lichter, 1982), a more efficient haploid system than the previously established anther culture. The application of a heat shock pretreatment (Keller and Armstrong, 1978), although originally discovered in anther culture, was the crucial step towards obtaining higher embryo frequencies in microspore embryogenesis. Another important step was the determination of specific developmental stages of potentially embryogenic microspores (Pechan and Keller, 1988; Telmer et al., 1992) and the application of colchicine to induce embryogenesis (Zhao et al., 1996) or to double chromosome number (Iqbal et al. 1994).

The highly efficient microspore culture system in *B. napus* has become an attractive model system for developmental (Fan et al., 1988; Zaki and Dickinson, 1991; Hause et al., 1993), biochemical (Pomeroy et al., 1991; Taylor et al., 1991), ultrastructural (Zaki and Dickinson, 1990; Telmer et al., 1993, 1995), physiological (Mandel, 1992; Hays, 1996) and molecular studies (Boutilier et al., 1994, Custers et al., 1996). Several studies revealed similarities between zygotic and MD embryos in *B. napus*. Microspore-derived embryos were morphologically similar to zygotic embryos (Yeung et al., 1996). They also accumulated seed storage products, such as proteins (Crouch, 1982) and lipids (Taylor et al., 1990, 1991; Pomeroy et al., 1991), similar to zygotic embryos. The response of MD embryos to abscisic acid (ABA) and osmoticum was similar to responses reported for zygotic embryos (Wilen et al., 1990) including rapid increases in storage protein transcripts and accumulation of oil-body proteins (Taylor et al., 1990; Wilen et al., 1990, 1991, Holbrook et al., 1991).

The major limitation of microspore embryogenesis in *Brassica* (and of microspore embryogenesis in general) is the genotype specificity. Routine production of a large number of MD embryos in *Brassica* is a reality mostly for var. Topas and very few other varieties of *B. napus*. Other genotypes are either recalcitrant or exhibit a relatively low frequency of embryogenesis. While the technique itself has been studied in great detail, resulting in

remarkable improvements over the years, a poor understanding of the fundamental aspects of haploid embryogenesis, in particular a lack of knowledge about events during the inductive phase, is the main barrier for overcoming the problems associated with this system.

1.5. Specific objectives of the study

In order to understand the process of microspore embryogenesis, it is of fundamental importance to understand the process of zygotic embryogenesis. A descriptive structural study of embryo development from the zygote, as occurs within maternal sporophytic tissue, provides an essential foundation for our better understanding of embryogenesis induced from the male gametophyte. This study was designed to investigate the details of MD embryo development in *B. napus*, providing a comparison to parallel events and stages in zygotic embryogenesis. Using this approach, the following research objectives were defined:

i.) to outline the major events during embryo and seed development *in vivo*,

ii.) to illustrate structural characteristics of the developing zygotic embryo as a basis for comparison to haploid MD embryos at the SEM and light microscope levels,

iii.) to examine the effect of an extremely reduced sucrose level, in a mannitol- or PEG-mediated low water potential environment, on the induction of microspore embryos, their subsequent development and the capacity to develop into haploid plantlets,

iv.) to describe in detail morphological changes during the entire time-course of microspore embryogenesis in *B. napus* induced on PEG and sucrose using SEM and to examine the differences in starch accumulation in sucrose and PEG embryos at the light microscope level,

v.) to compare the morphology of zygotic embryos developed within seeds and MD embryos,

vi.) to examine the effect of applied ABA and osmoticum on MD embryos during *in vitro* maturation and desiccation and on their subsequent acclimatization in an *ex vitro* environment.

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Section 2. TIME COURSE OF EMBRYO AND SEED DEVELOPMENT

2.1. Introduction

Alternation of two generations, a dominant sporophyte and a largely reduced minute gametophyte, takes place in the life cycle of vascular plants. The function of the gametophyte is to produce gametes during the haploid phase of the life cycle; when that is accomplished with adequate provision to ensure the union of egg and sperm, a zygote is formed and the sporophytic phase of the cycle begins again. In angiosperms, the sporophytic generation is triggered by the event known as double fertilization, a characteristic feature (with rare exceptions) of the reproduction of flowering plants. One of the two sperm cells fuses with the egg cell to form a diploid zygote, while the second sperm cell fuses with the two polar nuclei of the central cell in the embryo sac to form a triploid primary endosperm nucleus. This nucleus is the precursor of the endosperm, a tissue specialized for the nutritional support of the embryo. Thus, double fertilization sets the stage for simultaneous development of the embryo and its supporting tissue, the endosperm. Embryonic development proceeds within the confines of the maternal tissue of the ovule, which becomes the seed coat surrounding the developing embryo and endosperm (Dumas et al., 1998).

Embryogenesis describes the subsequent period of development, during which the zygote undergoes a complex series of morphological and cellular changes resulting in the formation of a developmentally arrested mature embryo. The events that occur during embryonic development establish the organization of the plant body and prepare the embryo for germination. Once the embryonic organ system is established, the embryo retains its developmental mode and precocious germination is suppressed by hormonal and environmental factors such as ABA level and restricted water uptake (Kermode, 1990; Black,

1991). Thus, an important role of the seed environment may be to maintain embryos in the developmental mode until they are fully formed and have accumulated sufficient reserves to permit subsequent germination and adequate growth of seedlings (Kermode, 1990). Although the levels of interaction between the developing embryo and surrounding tissues are not fully understood, plant hormones certainly play an important role in these processes, since levels and activities of various hormones change dramatically during embryo development in the embryonic and extraembryonic tissues as well as in the maternal sporophytic environment (Rock and Quatrano, 1995). The terminal phase of embryo development, desiccation or maturation drying (Kermode et al., 1986), is characterized by a rapid decline in water content and a rapid decrease in ABA accumulation. The seed becomes desiccation tolerant and upon desiccation enters into a metabolically inactive stage. The majority of seed species retain their viability when dried; in fact drying is a normal, final phase of maturation for "orthodox" seed (Bewley and Black, 1994). These terminal events of seed and embryo development make a sharp distinction between embryo development and all the postembryonic events (Steeves and Sussex, 1989). Upon imbibition of seeds, the germinative events are triggered and rapid mobilization of stored reserves as carbon and nitrogen sources occurs during early growth of the seedling.

One of the most comprehensive studies of angiosperm embryogeny was conducted three decades ago in *Capsella bursa-pastoris*, by Schulz and Jensen (1968a, 1968b, 1971). More recently, detailed histological studies of embryo development included two other model plants: *Arabidopsis thaliana*, for molecular genetics studies (Mansfield and Briarty, 1991a, 1991b), and carrot, for somatic embryogenesis studies (Lackie and Yeung, 1996). In *Brassica napus*, a model plant for studies of microspore embryogenesis, embryo development was described at the light microscope level using paraffin sections in a very detailed and truly remarkable work by Tykarska (1976, 1979, 1980, 1982, 1987a, 1987b). Although this work is still a masterpiece of descriptive embryogeny in dicots, with the technical advances in microscopical research there is a growing demand for more contemporary structural studies

on embryo development in this important oilseed species.

The purpose of the present section is to describe the major events during the development of the embryo within its maternal tissue (*in vivo*) as a basis for subsequent comparison to non zygotic embryogenesis *in vitro*. The identification of developmental stages during early embryo development and during seed maturation in *B. napus* was conducted with the light microscope using Historesin and Epoxy embedding techniques, and with the scanning electron microscope (SEM).

2.2. Materials and Methods

2.2.1. Growth chamber conditions for plants

Seeds of *Brassica napus* L. cv. Topas, line 4079 (courtesy of Dr. W. Keller, Plant Biotechnology Institute, NRC Saskatoon, Canada) were germinated in Jiffy 7 peat pellets every two weeks during two months to provide the plant material for the complete time course of embryo/seed development. Sets of 3-4 seedlings were potted in 8 inch pots biweekly and transferred to a growth chamber at 22/15°C (±2°C) day/night and 16 h illumination. Plants were watered and fertilized with 0.35 g L^{-1} Plant Prod® (15-15-18) nutrient solution twice each week. Flowers were hand-pollinated and tagged (at the same time) to indicate days after anthesis (DPA). Developing seeds from the siliques from the main inflorescences were collected for dissection and further processing of zygotic embryos for light microscopy. The seed moisture content was determined as a percentage on a fresh weight basis (mg/seed), as defined by the International Seed Testing Association (see Bewley and Black, 1994). Once the first set of plants had dry light brown siliques, fresh weight of the samples, collected for every second day from 8 DPA to 64 DPA, was measured. Each sample contained 20 seeds selected from the central portion of at least three siliques from the main inflorescences. Dry weight was measured after drying for 48 hours in an oven at 95°C.

2.2.2. Dissection of zygotic embryos from developing seeds

Dissection of zygotic embryos from developing seeds was carried out in a 10% sucrose solution. In the case of very small embryos (<300 μm), seeds were opened along the micropyle-hilum plane with a sharp hypodermic needle, and embryos with the entire suspensors and some surrounding tissue were dissected out. Pasteur pipettes were melted over a flame to make very fine hollow tips; these were used to carefully clean the cellular debris around the embryos by microcapillary force. Embryos were then pipetted into 1.5 ml Eppendorf tubes, fixed and further processed for SEM observation.

2.2.3. Fixation and processing of plant material for SEM

Dissected zygotic embryos were fixed sequentially in 1% and 3% glutaraldehyde in 0.025 M phosphate buffer (pH 6.8) prepared in 10% sucrose. Samples were postfixed overnight in 1% osmium tetroxide at 4°C, washed in distilled water 3-4 times and dehydrated in a graded acetone series (Fowke, 1995). Critical point drying (CPD) of extremely small samples (50-300 μm) was carried out in specially adapted capsule baskets, placed directly in the specimen trough of the critical point dryer. These baskets were made by cutting off the sloped portion of Beem embedding capsules (size 00, J.B. EM Services Inc.) so that lids would fit both ends. Each lid was punched out with a hole-punch and fitted with 50μm Nitex nylon mesh discs, 10 mm in diameter. Up to six of these baskets could stand upright in the trough of the Polaron E3000 Critical Point Dryer. After CPD, samples were mounted on SEM stubs using double-sided tape, sputter-coated with gold in an Edwards Sputter-Coater (Model S150B) and examined in a Philips 505 Scanning Electron Microscope at 30 kV. Images were recorded on Polaroid 665 film. Mounted photographic plates were scanned and the final images were prepared using Adobe Photoshop 4.0.

2.2.4. Fixation and processing of plant material for light microscopy - Araldite embedding

For light microscopy, punctured seeds (up to 13 DPA) or chopped pieces of dissected zygotic embryos were fixed as described for SEM. Samples were dehydrated in a graded ethanol series with 10% increments (Fowke, 1995); absolute ethanol was gradually replaced by propylene oxide (0°C). Tissue was infiltrated with Araldite embedding resin mixed with propylene oxide (1:1) at room temperature over a 6 hour period. Vials were covered with perforated aluminum foil and left in a fume hood overnight to allow evaporation of propylene oxide. Then, samples were transferred to fresh Araldite resin mixture in plastic embedding moulds and polymerized at 60°C for 48 h. Semithin sections (0.5-1.5 μm) were cut on a Reichert ultramicrotome with a freshly prepared glass knife. Sections were stained with Toluidine blue O (O'Brien and McCully, 1981), mounted in immersion oil and covered with 0.17 mm cover slips. A Zeiss Axioplan Universal epifluorescence photomicroscope was used and photographs were taken on Kodak T-max 100 ASA film. Negatives were scanned using a Polaroid SprintScan 35 Plus scanner and plates were prepared using Adobe Photoshop 4.0.

2.2.5. Fixation and processing of plant material for light microscopy and histochemical staining - Historesin embedding

Small seeds (up to 25 DPA) dissected from siliques were punctured carefully with a hypodermic needle to facilitate fixation, while larger seeds (30 DPA and older) were trimmed along an imaginary line connecting hilum and micropyle (leaving the embryo axis intact), removing 1/5 of the ovule from each side. The seeds were immediately fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.05 M phosphate buffer (pH 6.8), under vacuum for 20-30 min, then at 4°C overnight. Plant material was dehydrated in methyl cellosolve for 24 h followed by several changes of absolute ethanol over 3 days. Tissue was gradually infiltrated at 4°C first with LKB Historesin (Leica Canada) infiltration solution and absolute ethanol (1:2, 1:1 and 2:1 ratios) followed by 2-3 changes of pure Historesin infiltration

solution over several days. Polyethylene glycol (PEG) 200 was added to the final embedding medium usually at a 15 : 1 : 0.6 ratio (infiltration solution : hardener : PEG 200) to facilitate proper ribbon formation during sectioning (Yeung and Law, 1987). Thick serial sections (3μm) were cut with Ralph glass knives on a JB-4A microtome (Sorvall). Periodic acid-Schiff's reagent (PAS) was used for histochemical staining of carbohydrates, followed by counterstaining with Amido Black 10B for proteins (Yeung, 1984). Slides were observed with a Zeiss Axioplan Universal epifluorescence microscope and photographed with Ektachrome 100 color slide film. Colour slides were scanned on a Polaroid SprintScan 35 Plus and plates were assembled using Adobe Photoshop 4.0.

2.3. Results

2.3.1. Time course of embryo/seed development

Seed development in *B. napus*, from pollination (day 0) to dry pale brown siliques containing black seeds, was completed in less than 10 weeks under controlled conditions of the growth chamber (Fig 2.1.a). A detailed classification of the developmental stages over time under the controlled conditions of the growth chamber was out of the scope of the present study. Instead, the entire period of embryo and seed development was divided into three main overlapping phases (Fig. 2.1.a) based on the measurement of seed moisture content and the descriptive morphological characteristics of the seed coat and embryo. The first phase, early seed and embryo development, also called histodifferentiation (in embryo development), started with the first division of the zygote and terminated with the formation of the linear cotyledonary embryo. Dramatic morphological changes took place during this stage. Two major embryonic organ systems, the embryo axis and cotyledons were defined and all three principal tissue systems were initiated (Fig 2.1.b, 16 DPA). The originally transparent and colourless embryo proper first turned greenish at the heart stage, gradually attaining green colour at the early cotyledonary stage, while the seed coat was light green at the same stage.

The maturation phase, commenced early in the third week after anthesis and was characterized by the accumulation of storage reserves in the embryonic organs leading to a rapid increase in their volume as the overlapping cotyledons and curved embryo axis fully expanded and folded back gradually filling the entire ovule (Fig 2.1.b, 30 DPA). Maturation lasted several weeks during which the colour of the seed coat changed from light green to dark green, then to greenish-brown and eventually turned completely brown. The embryo axis changed from light green to green, then soon to yellowish-green and finally reached a pale yellow colour at the end of maturation. During the final stage, late embryogenesis, the embryo and seed prepared for desiccation, gradually became desiccated (with a moisture content of 7.5 %) and entered into a metabolically inactive or quiescent state. Dry brownish siliques contained desiccated seeds with the seed coat ranging from dark brown to black, while the embryo axes were whitish with pale yellow cotyledons.

Figure 2.1.a. Time course of embryo/seed development in *Brassica napus* L. Light micrographs of developing seeds (embedded in Historesin) at the onset of maturation (left, 16 DPA), during mid maturation (center, 30 DPA) and at the end of maturation (right, 48 DPA). Moisture content (%) measurements based on 20 seeds/sample.

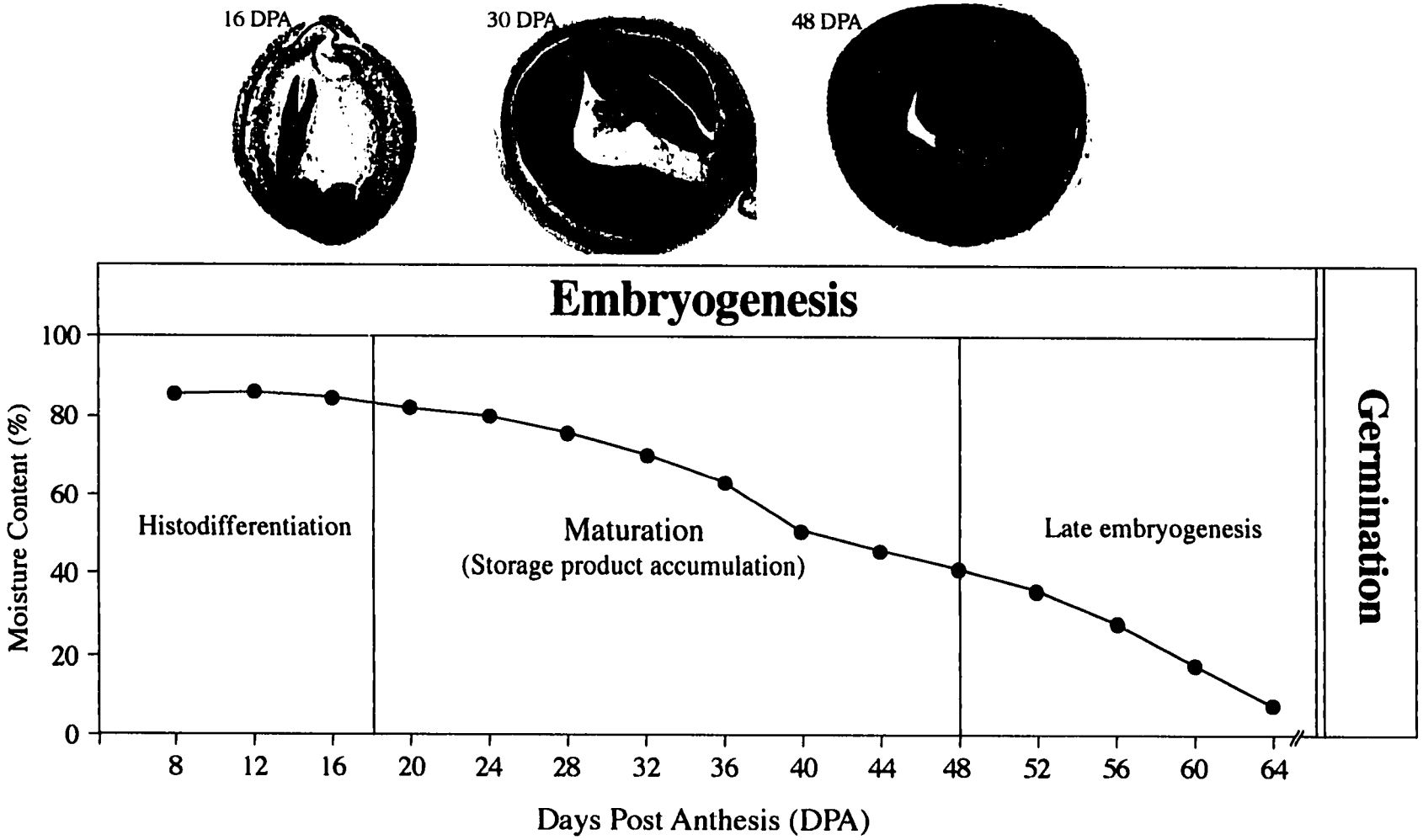
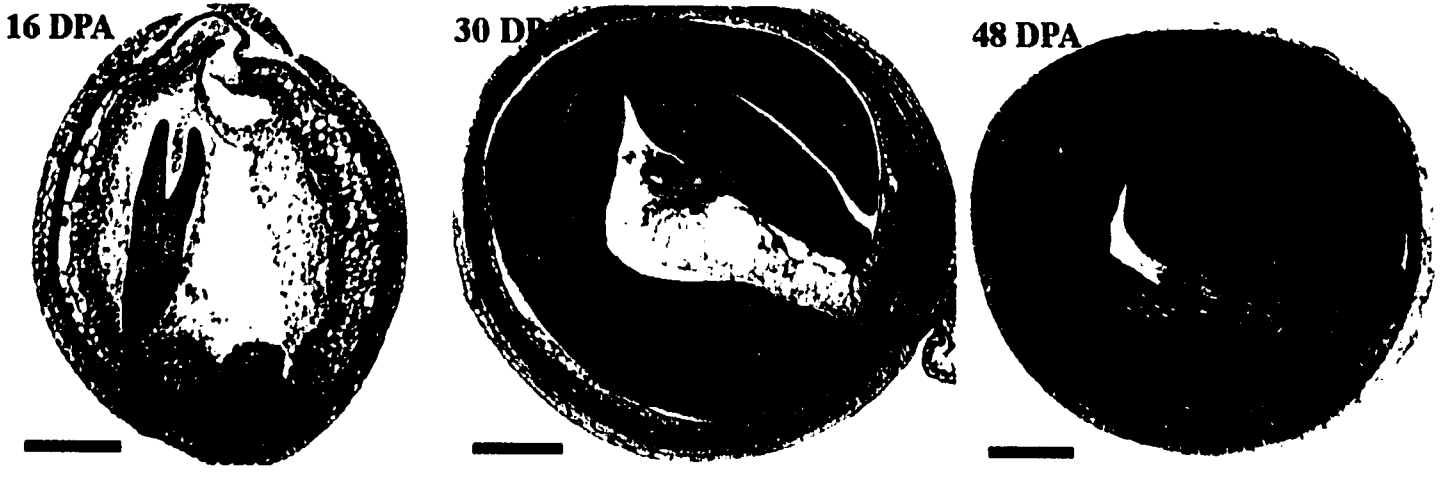

16 DPA
30 DPA
48 DPA
Embryogenesis
Germination
Moisture Content (%)
100
80
60
40
20
0
Histodifferentiation
Maturation
(Storage product accumulation)
Late embryogenesis
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
Days Post Anthesis (DPA)

Figure 2.1.b. Light micrographs (enlarged from Fig. 2.1.a) of developing seeds in *Brassica napus* L. at 16 DPA (left), 30 DPA (center) and 48 DPA (right).


16 DPA
30 DP
48 DPA

2.3.2. From ovules to seeds

After fertilization, the ovules became young seeds growing within the silique, which in the family Brassicaceae is an elongated fruit developed from the ovary. The developing seed in *B. napus* at 5 DPA (Fig. 2.2) consisted of:

i.) diploid embryo, developed from the zygote, (result of the fusion of egg cell and sperm cell),

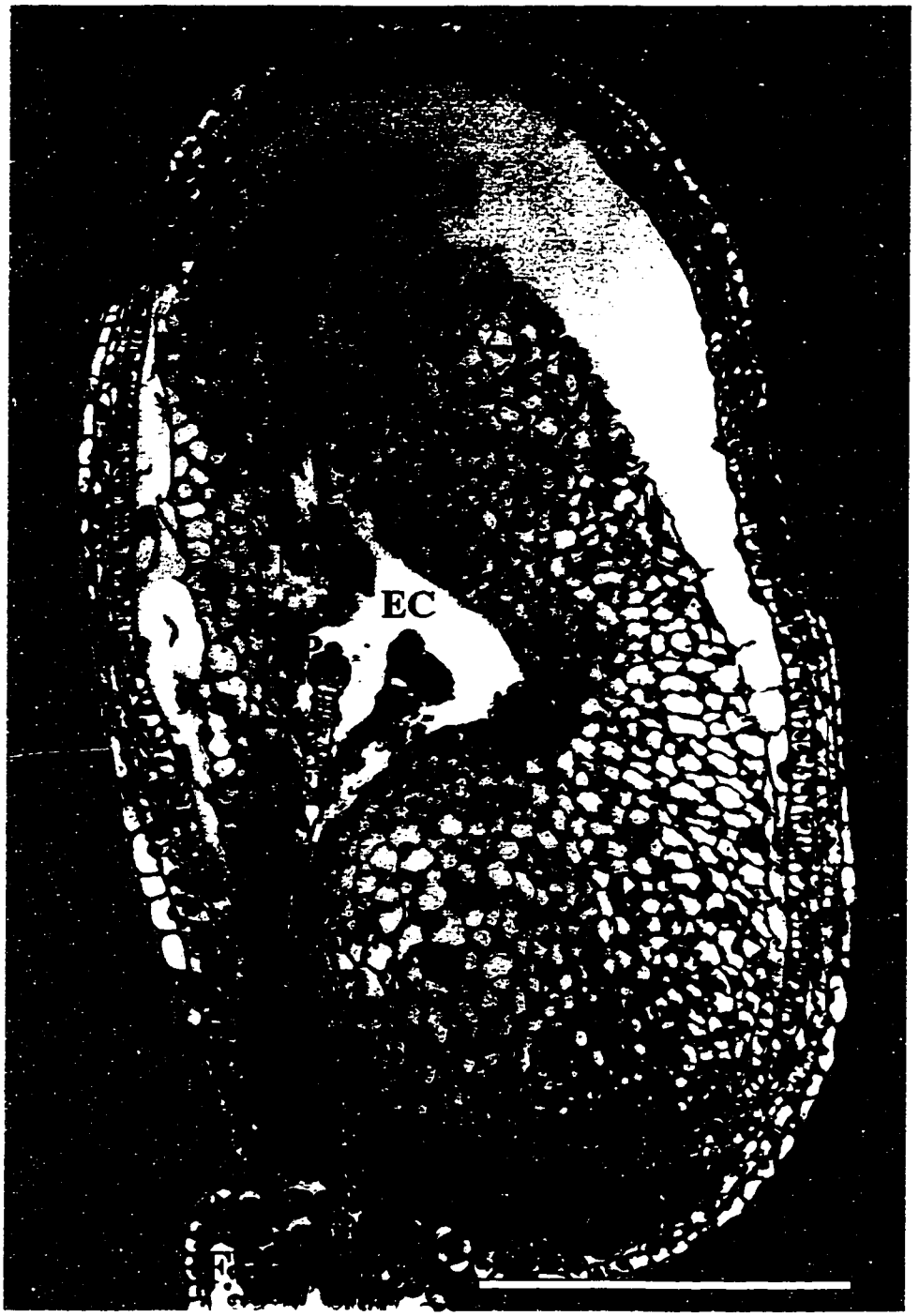
ii.) triploid nuclear endosperm, which arose after the two polar nuclei of the central cell fused with the other sperm cell,

iii.) chalazal proliferating tissue developed from the nucellus, the maternal tissue remaining within the ovule,

iv.) seed coat or testa, the protective layer surrounding the embryo formed from both integuments around the ovule.

Thus, the seed carries elements of the new diploid generation of the sporophyte, i.e., the embryo, triploid endosperm and elements originating from the maternal tissue (nucellus and seed coat). During early embryogeny (Fig 2.2), the initial formation of free-nuclear endosperm, which occupied a large portion of the endosperm cavity, was followed by the formation of the cellular endosperm (Fig 2.1.b, 16 DPA). Nutrients from endosperm cells were gradually absorbed by the developing embryo, and consequently the maturing seed had very few endosperm cells left (Fig 2.1.b, 30 DPA). At full maturity, the seed of *B. napus* had no endosperm and all the storage products were deposited in the large cotyledons and embryo axis (Fig 2.1.b, 48 DPA). The chalazal proliferating tissue (Fig 2.1.b, 16 DPA) (dark green in colour in fresh dissected seed) persisted throughout early seed development, eventually degenerating during early maturation. The outer and inner integuments, consisting of few cell layers at early seed development (Fig 2.1.b, 16 DPA) developed into the seed coat, which was eventually reduced to one or two distinct layers at the end of the maturation period (see Fig 2.1.b, 48 DPA).

Figure 2.2. Longitudinal section of developing seed (embedded in Araldite) in *Brassica napus* L. cv. Topas (5 DPA). Globular embryo proper (EP), suspensor (SU), endosperm cavity (EC), nuclear endosperm (NE), funiculus (FU), micropyle (M), seed coat (SC). Scale bar = 400 μm.


EC

2.3.3. Morphology of zygotic embryos dissected from developing seeds, as revealed by light microscopy and SEM

After the first asymmetrical transverse division of the zygote, the small apical cell (Fig 2.3.A, arrow) and elongated basal cell were produced. The elongated and highly vacuolated basal cell soon divided transversely giving rise to the suspensor, while the apical cell first underwent longitudinal divisions (quadrant stage) followed by transverse divisions to form the octant stage proembryo (3 DPA) (Fig 2.3.B). The resulting transverse walls (Fig 2.4.A, arrow) delineate the O′ boundary that can be followed throughout embryo development (Tykarska, 1976, 1979). This boundary separates the upper cell tier that will give rise to the cotyledons and embryo shoot apex, from the lower tier which will form the bulk of the embryo axis, i.e., hypocotyl and a portion of the embryonic root apex. The proembryo itself, less than 30 μm in diameter, was attached to a conspicuous filamentous suspensor consisting of a single file of seven cells (Fig 2.3.B, and 2.4.A). The elongated and narrow basal cell of the suspensor, which was confined within the surrounding maternal tissue within the ovule, anchored the proembryo to the micropylar region (Fig 2.3.B). At the early globular stage (Fig 2.3.C), the spherical proembryo was less than 50 μm in width and the suspensor consisted of nine cells (5 DPA). As a result of periclinal divisions of the octant cells, the protoderm layer (Fig 2.3.C, arrowhead) was already formed. The hypophysis, conspicuous uppermost suspensor cell (Fig 2.3.C, arrow), was also differentiated at the early globular stage. This cell and its derivatives (Fig 2.3.D, arrow) gradually became incorporated into the body of the embryo proper, giving rise to a part of the embryonic root apex. At the mid-globular stage, all three principal tissues (dermal, vascular and fundamental) were already initiated (Fig 2.3.D). During the globular and heart stage, free-nuclear endosperm filled the micropylar 'chamber', surrounding the suspensor (Fig 2.3.C, D and E) and adhering to the surface of the embryo proper (Fig 2.3.E, arrowhead).

A dramatic change from axial to bilateral symmetry of the embryo proper occurred at the

onset of the transition from globular to heart stage (Fig 2.4.C). The embryo proper expanded slightly in girth as a result of oriented cell divisions, attaining a gently ellipsoid shape in top view (not shown), and became flattened on its terminal region (Fig 2.4.C, arrow). Further outgrowth of the distal lobes (Fig 2.3.E, arrows) and apical-basal elongation resulted in the formation of heart-stage embryos (Fig 2.4.D, E) with two major embryonic organ systems, the embryo axis and the cotyledons already defined, as shown in Fig 2.3.F (11 DPA). Thus, the basic body plan of the embryo was already established at the heart stage when the embryo proper was approximately 120-150 μm in length. Considerable elongation of the cotyledons and embryo axis gave rise to a torpedo stage embryo (Fig 2.4.F) of approximately 400 μm in length. As rapid elongation of the embryo progressed, the cotyledons soon became flattened, and a linear embryo axis, consisting of shoot apex, hypocotyl and root apex reached about 0.70 mm in length (Fig 2.4.G). Therefore, this early cotyledonary stage was already attained at 13 DPA. Further changes occurred as the embryo entered the maturation period during which the curved cotyledons and embryo axis fully expanded, gradually filling the entire ovule and eventually crushing the suspensor cells. The two cotyledons overlapped, and folded back to enclose the curved embryo axis (Fig 2.4.H).

Figure 2.3. Light micrographs of *Brassica napus* L. zygotic embryos developed within the seeds (Araldite embedded). A. Apical cell (arrow) and elongated basal cell after the first asymmetrical division of the zygote. B. Proembryo (PE) at octant stage (3 DPA). Note long filamentous suspensor (SU). C. Early globular stage proembryo (PE) with already established protoderm layer (arrowhead) at 5 DPA. Note the hypophysis (arrow). Scale bars (A-C) = 50 μm. D. Globular stage embryo proper (EP) (7 DPA) with all three tissue systems: protoderm (arrowhead), procambium and the ground tissue. Procambial cells (PC) were more elongated than the ground meristem cells (double arrows). E. Heart stage embryo (9 DPA) with emerging cotyledonary lobes (arrows). Note thin layer of endosperm surrounding embryo proper (arrowhead). F. Late heart stage embryo (11 DPA): elongating cotyledons (CO), embryo axis (EA) with shoot apex (SA) and root apex (RA). Scale bars (D-F) = 100 μm.

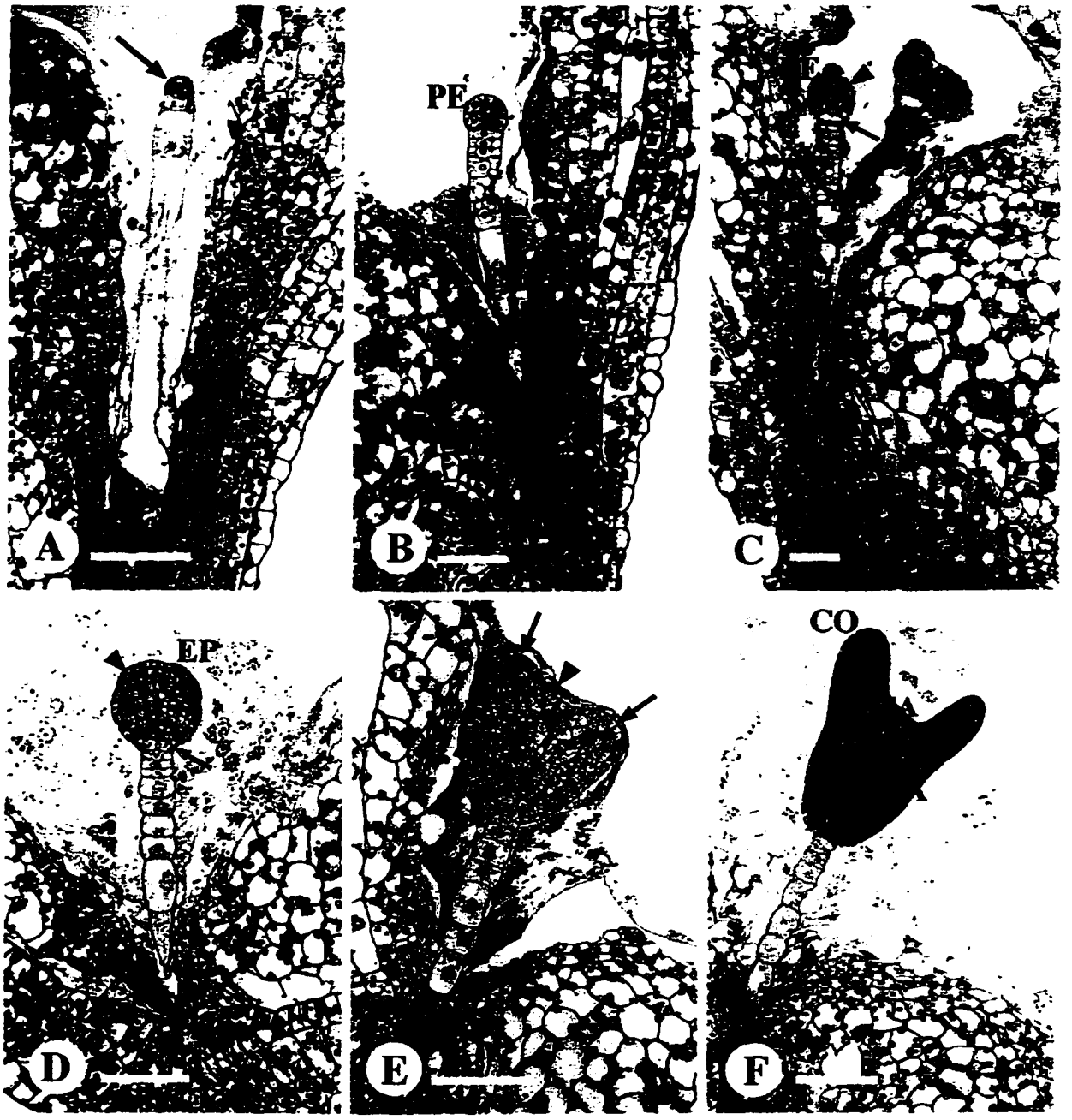
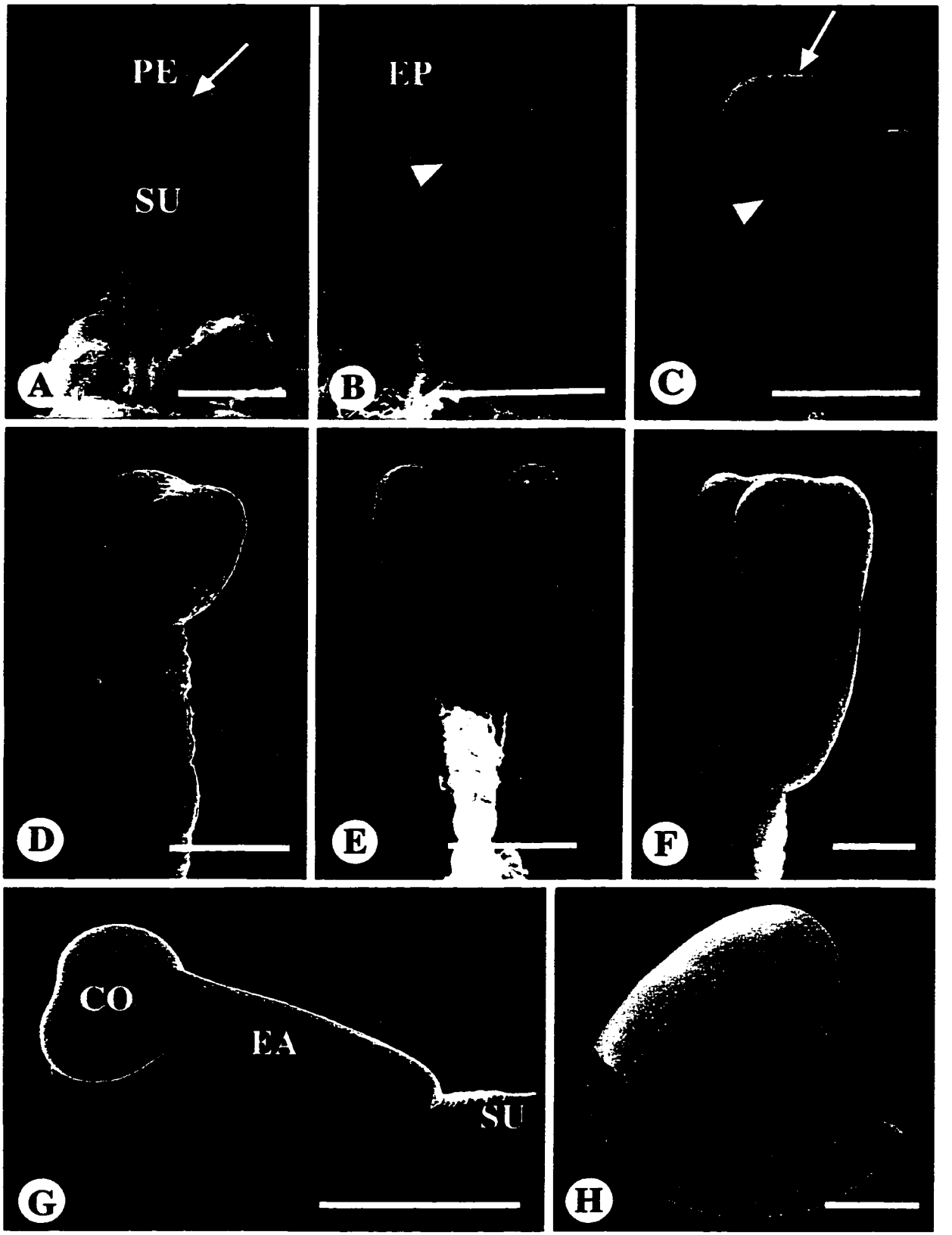

A
PE
B
C
EP
D
E
CO
F

Figure 2.4. Scanning electron micrographs of *Brassica napus* L. embryos dissected from seeds. A. Proembryo (PE) at octant stage with a suspensor (SU) containing 7 cells. Transverse division of quadrant cells delineates the O′ boundary (arrow) - this 'line' demarcates the upper tier that will give rise to shoot apex and cotyledons from the lower tier that will form hypocotyl and part of the embryonic root apex. Scale bar = 50 μm. B. Globular stage embryo proper (EP). Note the derivative(s) of the hypophysis (arrowhead). C. Embryo at transition from globular to heart stage exhibiting flattened apical region of the embryo proper (arrow) (8 DPA). Hypophysis derivatives indicated by arrowhead. D. Early heart stage embryo (9-10 DPA). E. Late heart stage embryo. F. Torpedo stage embryo (11 DPA). Scale bars (B-F) = 100 μm. G. Linear cotyledon stage embryo (13 DPA) with cotyledons (CO) and embryo axis (EA). Note the suspensor (SU). H. Curved embryo at the end of maturation (48 DPA). Scale bars (G-H) = 500 μm.


PE
SU
A
EP
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D
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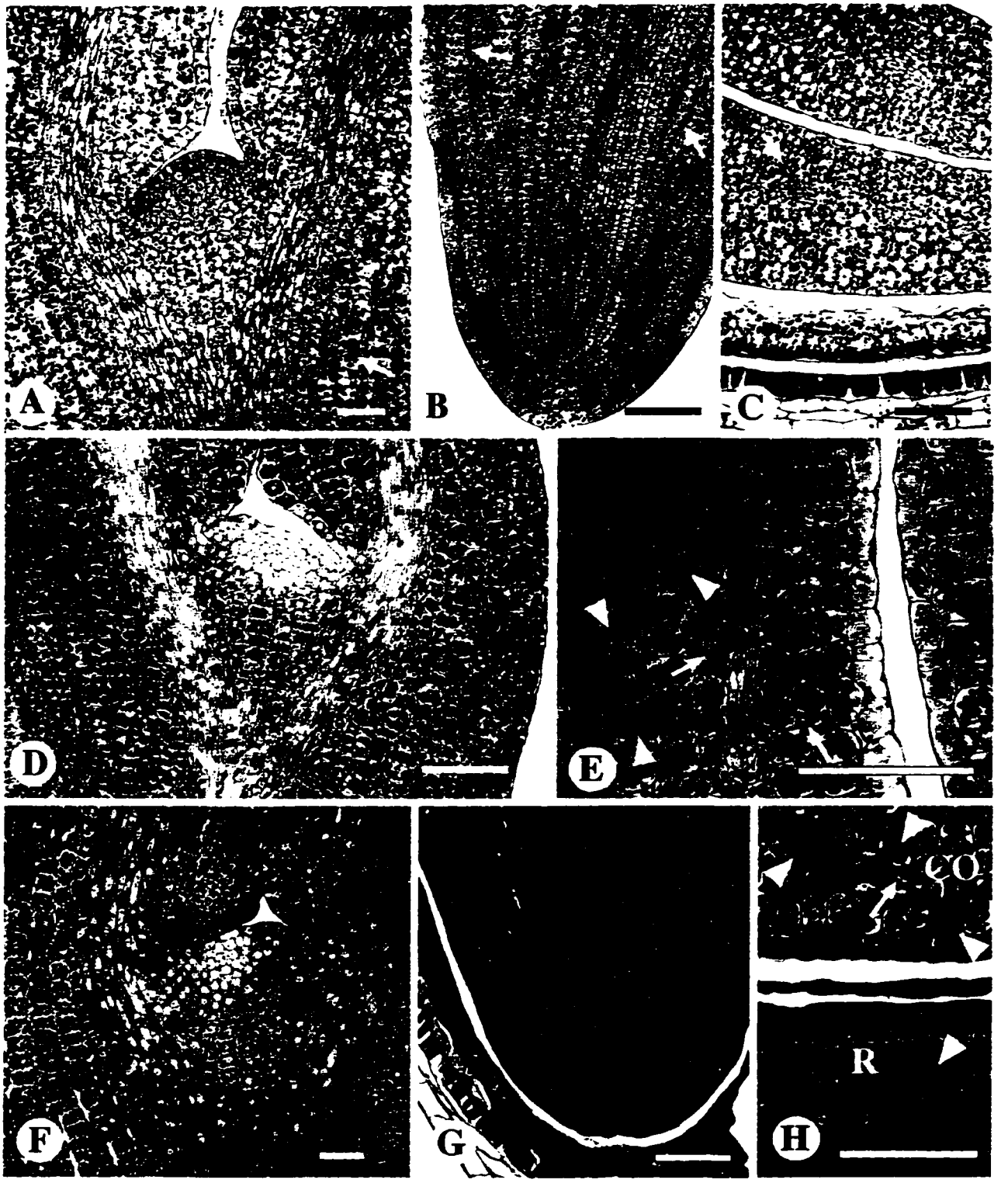
CO
EA
SU
G
H

2.3.4. Storage product accumulation during seed maturation - histochemical study

During early maturation (24 DPA) both the embryo and seed coat were green. The embryo axis and cotyledons were no longer linear, having started to bend gradually toward the chalazal end of the embryo sac. No protein bodies were present at this stage (Fig 2.5.A), while PAS-positive starch grains were found in most of the cells, with particular abundance in the cortex of the embryo axis and in the cotyledons (Fig 2.5.A, B, C, arrows). At mid maturation (36 DPA), multiple protein bodies (stained blue with Amido black 10B) were visible in the cortex of the embryo axis and in the cotyledons (Fig 2.5.D and E). Multiple starch granules (Fig 2.5.E, arrows) were still present in most of the embryonic cells. At the end of maturation (48 DPA), the cells were packed with numerous protein bodies (Fig 2.5.F, G and H). Most of the starch grains were already depleted (with the exception in the columella region of the root cap, Fig 2.5.G), and only a few remaining starch grains were found in the cotyledons (Fig 2.5.H, arrow). Myrosin cells, protein accumulating idioblasts containing the enzyme myrosinase (Thangstad et al., 1990; Höglund et al., 1991), were stained faintly compared to the cells filled with the two main storage proteins, napin and cruciferin. Myrosin cells were observed at mid maturation (Fig 2.5.E, yellow arrowhead). By the end of maturation (48 DPA) they were rather distinct in cortical cells of the radicle and in the cotyledons (Fig 2.5.H, yellow arrowheads).

Figure 2.5. Histochemical observations of protein bodies and starch grains in embryonic organs of *Brassica napus* L. during seed maturation *in vivo* (embedded in Historesin). A-C. Early maturation (24 DPA). A. Shoot apex region. Cortical cells are filled with starch grains, stained dark purple (arrows). B. Embryonic root at the same stage. Numerous starch grains in cortical cells (arrows). C. A portion of two curved cotyledons with the seed coat. D. Embryonic shoot apex (36 DPA). E. A portion of two cotyledons during mid maturation stage. Note protein bodies (white arrowheads) and individual myrosin cell (yellow arrowhead). Starch grains stained dark purple (arrows). F. Longitudinal section of the shoot apex and G. embryonic root at 48 DPA (end of maturation). H. Enlarged detail of cotyledon (CO) and cortex of radicle (R) at the same stage. Myrosin cells stained light blue (yellow arrowheads). Note numerous protein bodies (white arrowheads). Only a very few starch grains remain (arrow). All sections were stained with PAS and Amido black10B. Scale bars = 100 μm.


A
B
C
D
E
F
G
CO
R
H

2.4. Discussion

Two basic methods for preparing plant material for light microscope investigation are commonly used in studies of plant anatomy. One involves embedding in paraffin, and another, which is currently widely used, involves superior fixation with aldehydes and embedding in plastic, such as Epoxy resin or Historesin. Tissue embedding in plastic, employed in this study, offers many advantages over the traditional embedding medium, paraffin. Most importantly, cells and tissues are better preserved after fixation with aldehydes with less shrinkage, while thin sections are routinely obtained with little or no knife damage (Fowke and Rennie, 1995). As a result, considerably more cell details are visible at the light microscope level, making plastic embedding a far superior technique for studies of plant structure. The SEM provides the unique opportunity for three-dimensional observation of external structure, and is particularly useful for visualizing surface-associated details of specimens, which otherwise cannot be achieved by light microscopy.

A detailed staging system of embryo development in *B. napus* (varieties Górczańska and Mlochowska), was originally elaborated by Tykarska (1980). Similar classifications of morphological stages were also described in alfalfa (Xu et al., 1991) and in *Vicia faba* (Borisjuk et al., 1995). For the purpose of the present study, development of the rapeseed embryo and seed was divided into three overlapping phases, similar to the description of general angiosperm embryo development by West and Harada (1993).

Development of the embryo in *B. napus* (Brassicaceae) followed the Onagrad Type, *Capsella* variation (Johansen, 1950). Rapeseed embryogeny resembled that of *C. bursa-pastoris* (Schulz and Jensen, 1968a, 1968b) with the exception of the shape of derivatives of the basal cell. The most basal cell was not large and swollen as in *Capsella*, but considerably elongated and narrow. Maheshwari (1950) proposed another embryonomic classification system in angiosperms, in which five types were described, and *B. napus* followed the division pattern

described as the Crucifer type. Both classification systems were based on the early division patterns in proembryo development, relying strictly on the embryonomic laws and formulae (Johansen, 1950). This traditional view that the fundamental organization of the embryo body is a direct result of a sequence of precise cell divisions during early embryogeny is no longer acceptable, in the era of extensive molecular genetic studies on embryogenic developmental mutants in *Arabidopsis* (Meinke, 1995; Jürgens et al., 1991; Laux and Jürgens, 1994). Nevertheless, these classification systems are still of great importance for taxonomical and phylogenetic interpretations (Steeves and Sussex, 1989).

The descriptive study of embryogeny in *B. napus* presented in this section confirms and extends the observations Tykarska (1976, 1979), who examined embryo development in *B. napus* using paraffin embedded specimens. The first asymmetrical division of the zygote gave rise to a small apical cell which underwent a precise set of divisions to form most of the embryo proper and a basal cell which divided transversely to form a filamentous single-filed suspensor with its uppermost cell - the hypophysis. This cell and its derivatives became incorporated into the body of the embryo, contributing, in part, to root apex formation (Tykarska, 1976, 1979). Therefore, a precise cell division pattern existed during zygotic embryogenesis of *B. napus* resulting in a linear cotyledon stage embryo with three spatial domains: apical domain (cotyledons, shoot apex), central domain (hypocotyl), and basal domain (root apex), as previously described for dicotyledonous plant embryos by West and Harada (1993). The boundary between the apical domain and the other two domains - transverse O' line - shown in Fig 2.4.A (also see 2.3.B) appeared as early as the octant stage of the proembryo (Tykarska, 1976, 1979). The transition stage (a 'switch' from axial to bilateral symmetry) has often been referred to as the triangular stage in studies of *Arabidopsis* embryogeny (see Jürgens et al., 1991; also Mansfield and Briarty, 1991a). In the present study, this distinct stage is referred to as the transition stage (Fig 2.4.C).

Rapeseed endosperm formation was of the nuclear type with the initial formation of free-

nuclear endosperm, followed by cellularization of the endosperm (van Lammeren et al., 1996), similar to *Arabidopsis* (Mansfield and Briarty, 1990a, 1990b). In this study, the micropylar region was occupied by free-nuclear endosperm at the early heart stage (see Fig 2.3.E), but at the late heart stage cellular endosperm was noticeable in the area surrounding the suspensor (Fig 2.3.F). Van Lammeren et al. (1996) also reported cellularization in the area surrounding the suspensor and embryo proper in *B. napus* at the heart stage. The expanding embryo absorbed most of the endosperm by 30 DPA (see Fig 2.1.b) and at full maturity the seeds of *B. napus* were nonendospermic, also called exalbuminous (Bewley and Black, 1994).

During seed maturation, transient starch accumulation occurred in the ground tissue of the rapeseed embryo, visualized as the large purple PAS-positive grains. The maximum deposition of starch grains occurred during early maturation (24 DPA), similar to previous reports by Tykarska (1982) and Yeung et al. (1996). The synthesis of the storage proteins napin and cruciferin, commenced approximately at the same time (Crouch and Sussex, 1982, Murphy and Cummins, 1989a, 1989b). These proteins were deposited into spherical protein bodies within cells, often called aleurone cells (Tykarska, 1982), which became more numerous and densely stained with Amido Black during the advanced stage of maturation (from 36 DPA to 48 DPA). At the end of maturation, starch grains in the embryonic organs were largely depleted, similar to reports in *B. campestris* (Leprince et al., 1990) as the main seed reserves of carbon and nitrogen were deposited in the form of storage lipids and proteins, the two principal seed storage products in *Brassica*. The synthesis of these storage reserves required a supply of suitable precursors and energy. The transient starch deposition during the first half of seed development is due to assimilates imported from the leaves at the peak of their photosynthetic activity (King et al., 1997; Da Silva et al., 1997). However, this starch was not sufficient for the growing demand in maturing seeds (Norton and Harris, 1975) and an additional import of assimilates was required once leaf senescence occurred. In the absence of leaves, the majority of assimilate demand in seeds was then supplied by photosynthesis of the silique walls (Norton and Harris, 1975; King et al., 1997).

This section has illustrated structural details from post-fertilization events, including the formation of apical and basal cells after the first division of the zygote, to the end of seed maturation, and provides an important framework for subsequent comparison to microspore embryogenesis, in Section 4.

2.5. Summary

The time course of embryo and seed development in *B. napus* was divided into three main phases: early seed and embryo development, also called histodifferentiation, the maturation phase and late embryogenesis. The first two phases were studied at the SEM and light microscope levels. The major morphological changes occurred during early embryogeny in which embryos proceeded through globular, heart, torpedo and cotyledonary stages, exhibiting a precise cell division pattern. Early embryo development was terminated with the formation of a linear cotyledonary embryo with two embryonic organs, embryo axis and cotyledons. The maturation phase was characterized by the accumulation of storage reserves in the embryonic organs leading to a rapid increase in their volume. Transient deposition of starch occurred in embryonic organs during early seed maturation, while filling of protein bodies took place later, during mid maturation, reaching a peak at the end of maturation (48 DPA). Late embryogenesis was a terminal phase during which embryo and seed gradually endured a substantial water loss, entering into a metabolically inactive or quiescent state.

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Section 3. INDUCTION OF MICROSPORE EMBRYOGENESIS IN WATER STRESS AND SUCROSE RESTRICTIVE CONDITIONS

3.1. Introduction

Among the factors that play a critical role in the induction of microspore embryogenesis in *Brassica* species, choice of a carbon source and its concentration are considered to be of particular importance. Commonly used protocols for microspore and anther culture in *Brassica* include sucrose (usually at concentrations of 8-17%) as an optimal and essential osmotic and nutritional component required for haploid embryo induction (Keller et al., 1975; Lichter, 1981, 1982; Dunwell and Thurling, 1985; Baillie et al., 1992; Ferrie et al., 1995). In anther culture of *Brassica campestris*, sucrose was superior to other compounds such as mannitol and sorbitol, both of which failed to induce embryogenic division of pollen (Hamaoka et al., 1991). However, excess sucrose provided in the liquid medium could lead to substantial sugar uptake by MD embryos resulting in abundant starch accumulation as observed by Rahman (1993) and Yeung et al. (1996). Moreover, zygotic embryos dissected from ovules at a torpedo stage and cultured on sucrose medium for two weeks accumulated starch grains in a similar manner (Rahman, 1993).

The use of sucrose-free medium in micropropagation of chlorophyllous explants, i.e., the concept of photoautotrophic micropropagation, was proposed by Kozai et al. (1988). Since then it has been found in several species that carbohydrates in the medium were not necessarily required when high CO_2 and high photosynthetic photon flux were provided (Kozai, 1991). The only success using sucrose-reduced or sucrose-free medium in pollen embryogenesis was achieved when an initial few-day-long starvation period was followed by transfer to carbohydrate-rich medium as reported in *Nicotiana* (Imamura et al., 1982; Kyo and Harada, 1985), barley (Olsen, 1991) and rice (Ogawa et al., 1994).

In the present study, efforts were focused on uncoupling osmotic from nutritional requirements for embryo induction, in the attempt to bypass potential abundant sugar uptake by embryos. Since high osmotic pressure of the culture medium was a requirement for microspore embryogenesis, non-metabolizable osmotica such as mannitol and polyethylene glycol (PEG) were applied as alternative osmotic agents in concentrations that provided osmotic potentials comparable to that generated by high sucrose in the medium.

Mannitol, which is commonly used as an external osmoticum, can easily penetrate cell walls but the plasmalemma is considered to be relatively impermeable to it (Rains, 1989), while high molecular weight PEG 4000 is too large to penetrate cell walls (Carpita et al., 1979; Rains 1989). A high concentration of mannitol has been used to induce osmotic stress in MD embryos of *Brassica napus* (Huang et al., 1991) and in microspore (A.M.R. Ferrie, personal communication) and anther culture of *Brassica campestris* (Hamaoka et al., 1991). Also, it has been used in the pretreatment for barley anther (Roberts-Oehlschlager and Dunwell, 1990) and pollen culture (Wei et al., 1986), tobacco anther (Imamura and Harada, 1980) and pollen culture (Imamura et al., 1982) and in wheat microspore culture (Hu et al., 1995).

Polyethylene glycol is a neutral polymer, highly soluble in water and nontoxic. In solution, PEG creates a more negative water potential, facilitating a water-restricted environment (Rains, 1989). High molecular weight PEG is frequently used in biological systems for various purposes. For instance, in horticulture it is used in a pre-sowing seed treatment called osmotic conditioning or priming (Bradford, 1986). It is commonly used in various *in vitro* studies to create a water stress environment (Soja and Soja, 1989). In plant protoplast culture it has a wide application in protoplast fusion (Kao et al., 1974; Kao and Michayluk, 1989) and in PEG-mediated protoplast transformation (Koop and Kofer, 1995). In somatic embryogenesis PEG was used as an osmoticum for embryo maturation in conifers (Attree et al., 1991). Recently, it has become accepted as an osmotic agent of choice in somatic embryogenesis of some angiosperm species, such as celery (Saranga et al., 1992), pecan (Burns and Wetzstein,

1995), horse chestnut (Capuana and Debergh, 1997) and *Hevea* (Linossier et al., 1997). Nevertheless, its application in anther and pollen culture has been rather limited. Use of PEG has been attempted in combination with sucrose in barley anther culture (Thörn, 1988) and in microspore culture in combination with Ficoll and glucose (Kao, 1993). Ilić-Grubor et al. (1997) first reported successful induction of microspore embryogenesis of *Brassica napus* using PEG as an osmoticum.

The objective of the experiments described in this section is to determine whether induction of microspore embryos, their subsequent development, and the capacity to develop into haploid plantlets is affected by extremely reduced sucrose level, in a mannitol- or PEG-mediated low water potential environment.

3.2. Materials and Methods

3.2.1. Growth condition of donor plants

Plants of *Brassica napus* L. cv. Topas line 4079 were grown in a growth chamber with a 20°/15°C day/night temperature regime and 16 h illumination provided by VHO (very high output) Sylvania cool white fluorescent lamps. Prior to bolting, the temperature was lowered to 10°/5° C (day/night), and plants were maintained at this regime providing water and nutrients twice per week with 0.35 g L^{-1} Plant Prod® (15-15-18) nutrient solution. Another set of plants was grown at 22°/15°C day/night temperature regime and 16/8 h photoperiod and hand-pollinated flowers were tagged to indicate days after anthesis (DPA). Immature zygotic embryos were dissected from developing ovules (14 and 15 DPA) and used for comparison with MD embryos.

3.2.2. Measurement of osmotic potentials of media

Osmotic potentials of sucrose, mannitol and PEG 4000 (w/v) in 1/2 NLN medium were determined using a vapor pressure osmometer (Model 5230A, Wescor Inc.). Milliosmoles (mOs/kg H_2O) were converted to MPa using the van't Hoff equation.

3.2.3. Isolation and culture of microspores

A mixed population consisting of microspores from mid- and late-uninucleate stages to young bicellular pollen was isolated from the flower buds using a protocol for *Brassica napus*, modified from that described by Ferrie and Keller (1995). For each treatment, twelve surface-sterilized buds (3.6-4.2 mm in length) were macerated in 5 ml half strength B5 washing medium (Gamborg et al., 1968) containing 13% sucrose (w/v) in 50 ml beakers. The crude suspension was filtered through 44 µm Nitex nylon mesh, both beakers and meshes were rinsed, and a total of 20 ml was collected into 50 ml centrifuge tubes and centrifuged at 150 g for 3 min. The pellets were resuspended in 5 ml of washing medium, the washing procedure was repeated twice, then the final supernatant was carefully pipetted out, leaving the pellet in approximately 0.3-0.4 ml of washing medium. Final resuspension was done by adding filter-sterilized half strength NLN medium (Lichter, 1982) containing 13% (w/v) sucrose as control treatment; in the other two treatments, 1/2 NLN sucrose-free medium with 8% (w/v) mannitol or 25% (w/v) PEG 4000 (Fluka Chemika) was added, respectively. Therefore, both 1/2 NLN-mannitol and -PEG medium contained only about 0.1% sucrose, remaining from the washing medium. Nalgene filter units were used for filter sterilization of the medium, with 0.8 µm (prefiltration) and 0.22 µm Millipore filter discs. Aliquots of 10 ml of microspore suspension were plated into 100 x 15 mm sterile petri dishes, with an approximate plating density of 40,000 microspores/ml. Plates were first incubated at 35°C for one hour followed by 33°C for the next 14-18 hours and then placed on a shaker (50 rpm) in the dark at 24°C for 12 days. During the first eight days in culture, PEG and mannitol media were gradually

diluted by adding to the plates an adequate volume of a simple ½ NLN medium, containing no sucrose, mannitol or PEG, to give a final concentration of approximately 22% PEG and 7% mannitol. The concentration of sucrose in the control treatment was lowered the same way to a final concentration of 11%.

Twelve days after isolation, plates with MD embryos were placed under light (2000 lux) with 16/8 hours photoperiod at 24°C for an additional week. About two weeks after isolation, a small volume (0.5 ml) of 2% sucrose in ½ NLN medium was added to the plates. Experiments dealing with mannitol as osmoticum were repeated three times with five replicates, while experiments dealing with PEG and sucrose as a control have been routinely performed for over two years.

3.2.4. Haploid plantlet formation and measurement of conversion frequency

Two, three and four-week-old sucrose and PEG embryos, all in at least torpedo or early cotyledonary stages, were placed on filter paper on a solid hormone-free B5 germination medium containing 1% sucrose into Pyrex® No 3140 glass containers (90 x 50 mm). Containers were placed in a culture room at 24°C with 16/8 hour photoperiod and 2000 lux illumination for four weeks. The conversion frequency was expressed as number of plantlets with both normal root growth and the first pair of true leaves, divided by the total number of embryos plated. The experiments were repeated three times with three replicates. Data are presented as the mean values with ± standard deviations as error bars.

3.3. Results

3.3.1. Induction of MD embryos in high sucrose, mannitol and PEG medium

In order to meet specific osmotic requirements for microspore embryogenesis in *Brassica*,

mannitol and PEG were applied as alternative osmotic agents in concentrations that provided an osmotic potential similar to that created by high sucrose. In the preliminary experiments, a wider range of concentrations for each osmoticum was tested for microspore culture, after which 8% mannitol (0.44 M) and 25% PEG (0.06 M) were chosen and used in the following experiments. The osmotic potentials of these solute concentrations were similar to that created by 13% sucrose (0.38 M), all three lying within a narrow range of about -1.1 MPa (Fig 3.1).

Induction of MD embryos depended on the type of osmoticum used: microspores cultured on sucrose and PEG medium developed into embryos within two weeks while mannitol-cultured microspores yielded no embryos. Although these microspores became swollen immediately after heat shock treatment, no divisions were observed and they were presumably dead within a week in culture on mannitol medium. After two weeks in culture, some of the PEG embryos were about 1-3 mm in length, reaching the cotyledonary stage; however, most of them were smaller, still in the globular, heart-shape or torpedo stages. When placed under light, these embryos quickly became green (Fig 3.2.A and C) and further increased in size, due to elongation of the embryo axis and further differentiation of cotyledons. An assessment of frequency of embryo formation was not attempted, due to the technical difficulty of counting PEG embryos of such a small size. Nevertheless, the number of microspores induced to undergo embryogenic development appeared comparable to that of the sucrose control (Fig 3.2.A), based on visual observation of microspores and developing embryos with the inverted light microscope. Three-week-old PEG embryos had well developed cotyledons, often widely opened and dark green, suggesting possible photosynthetic activity (Fig 3.2.F). MD embryos on sucrose medium, when compared to both zygotic and PEG embryos, were much larger and had elongated embryo axes and relatively small underdeveloped cotyledons (Fig 3.2.D).

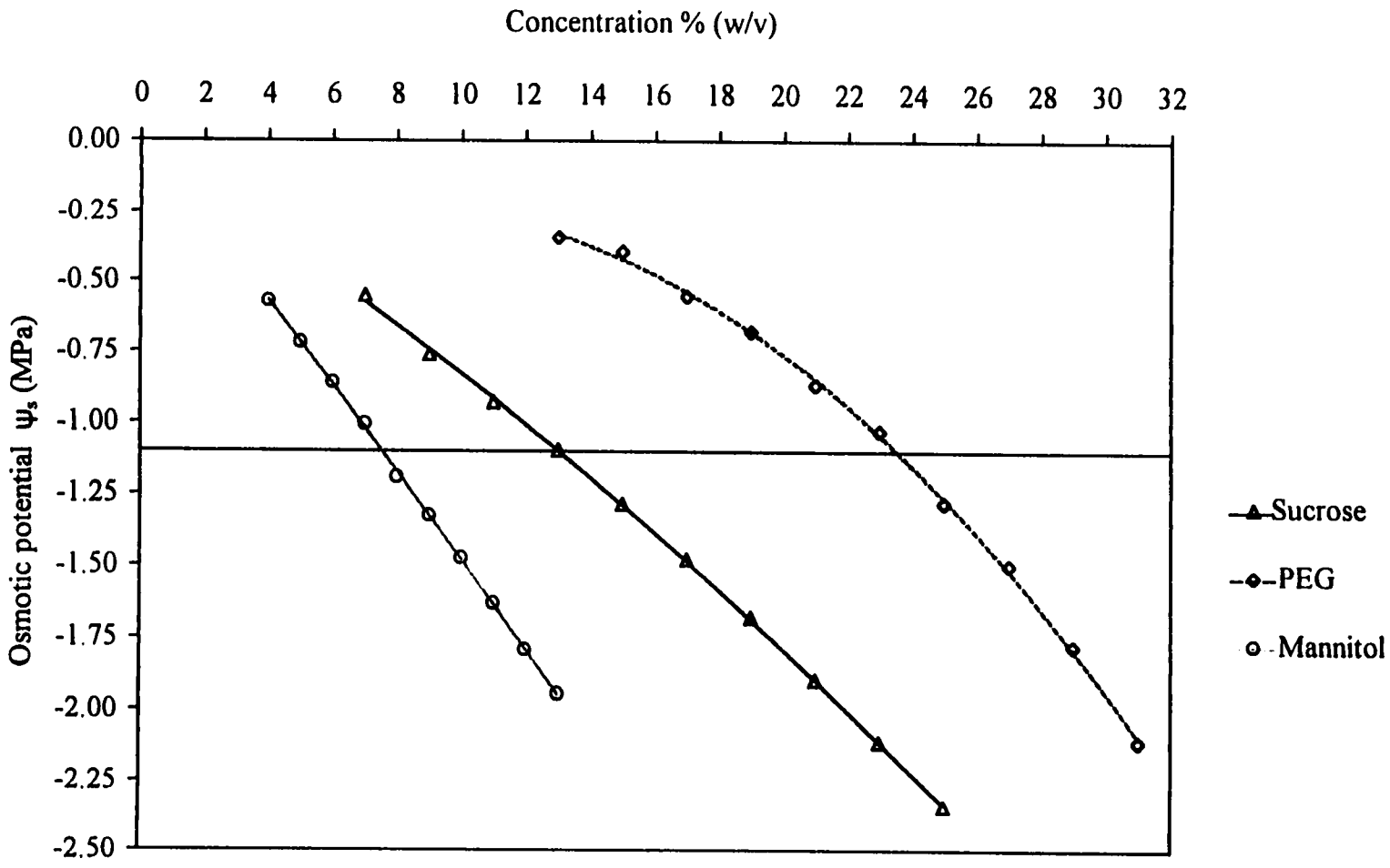
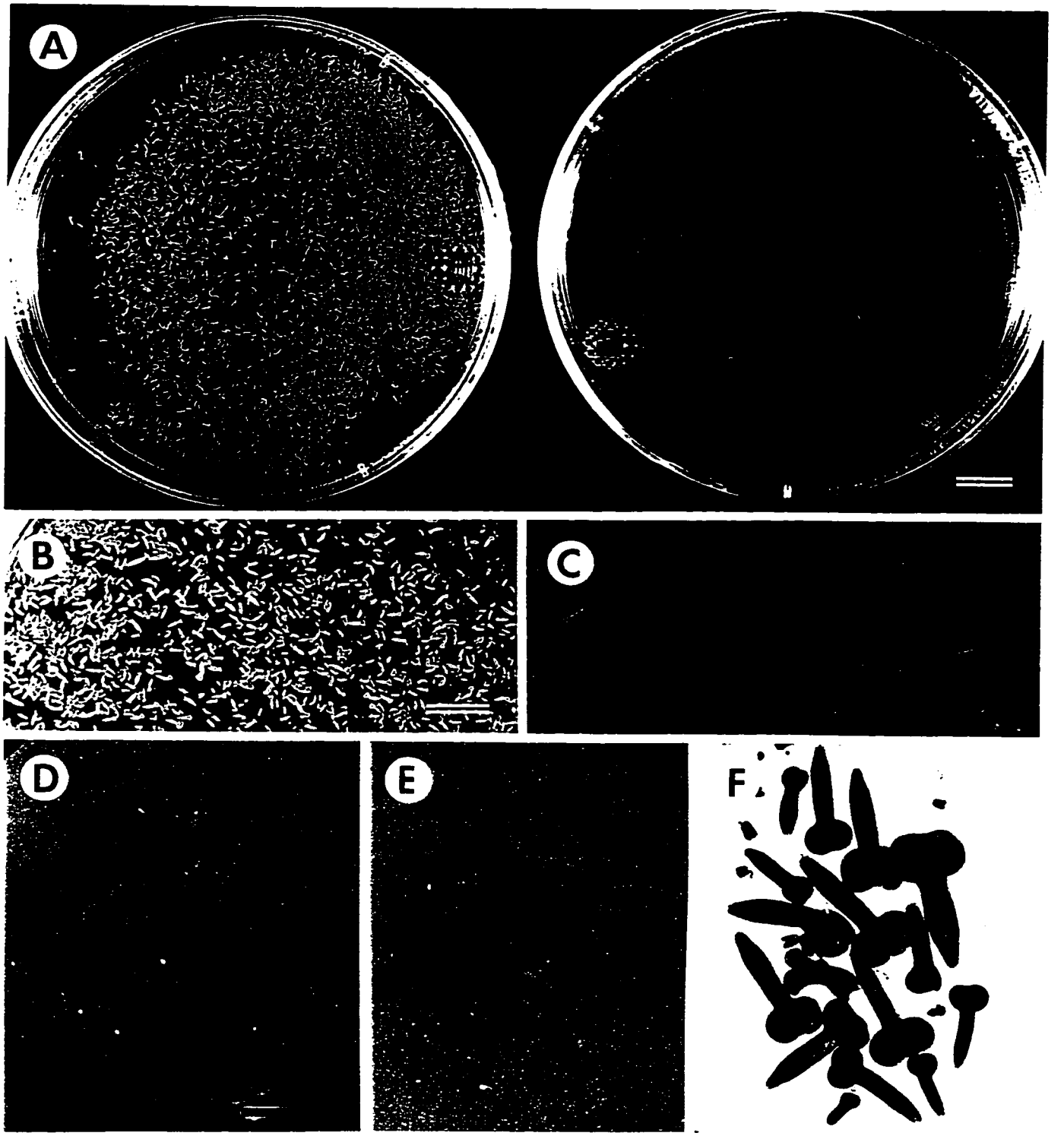


Figure 3.1. Osmotic potentials of sucrose, mannitol and polyethylene glycol 4000 in 1/2 NLN medium

Figure 3.2.A-F. A. Sixteen day-old microspore-derived embryos of *Brassica napus* L. on different types of osmoticum: sucrose (left) and polyethylene glycol 4000 (right). Plates were kept under the light for two days. Bar = 10 mm. B. Enlargement of sucrose embryos (from A). C. Enlargement of PEG embryos (from A). (B-C) Bar = 5 mm. D. Three-week-old sucrose embryos. E. Zygotic immature embryos developed within seeds dissected at 14 and 15 DPA. F. Three-week-old PEG embryos. (D-F) Bar = 1 mm.

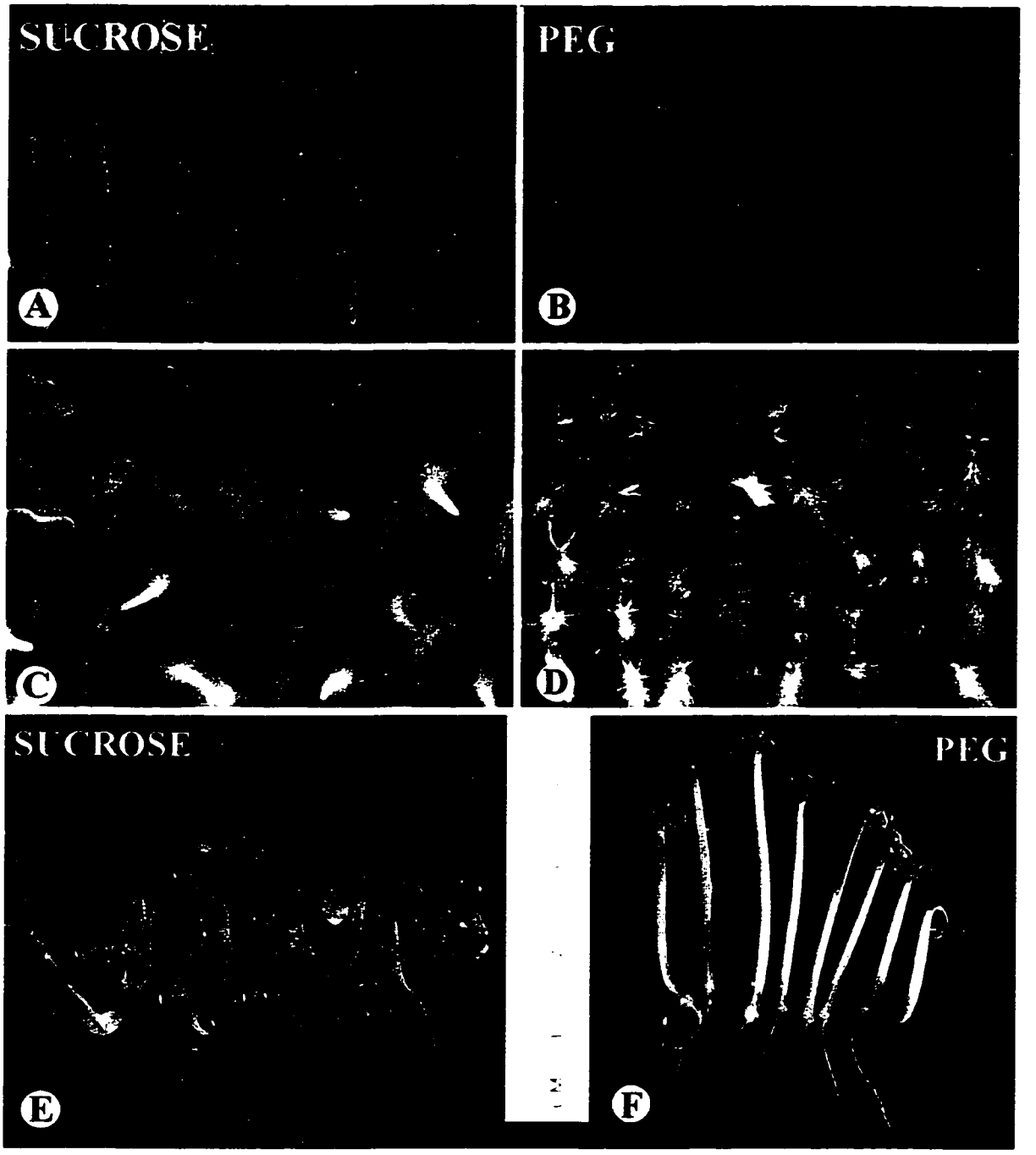

A
B
C
D
E
F

3.3.2 Germination of MD embryos and plantlet formation *in vitro*

Despite the difference in embryo size and morphology between sucrose and PEG embryos (Fig 3.3.A and B), once the embryos were placed on solid germination medium, the initial events (during 48 hours) proceeded identically in both groups. The majority of MD embryos from both treatments had elongated radicles with numerous root hairs (Fig 3.3.C and D), except for a few that were damaged during manipulation. Within 2-3 weeks, haploid plantlets were formed in both groups. At this time, a considerable difference between sucrose and PEG plantlets was noticeable. Plantlets formed from sucrose embryos had stunted and thick hypocotyls and aberrant and swollen cotyledons (Fig 3.3.E). After a prolonged period in culture, these hypocotyls often developed callus bearing numerous secondary embryos (Fig 3.4.A-C). In contrast, plantlets from PEG embryos had very elongated and thin hypocotyls (Fig 3.3.F) and only occasionally secondary embryos were formed on these hypocotyls. While growth of embryonic roots was vigorous at each time interval tested in both sucrose and PEG embryos, formation of first true leaves was slow and delayed in both groups. Instead of true leaves, PEG plantlets frequently bore a second set of cotyledons (Fig 3.4.D). Histological analysis of shoot apices at the light microscope level revealed normal shoot apical meristems in both groups (data not shown).

Plantlet conversion frequency (%) was very similar in both sucrose and PEG embryos at each time interval (Fig 3.5). While the elongation of hypocotyls and formation of a root system occurred within two weeks in most MD embryos, the formation of a shoot system was either lacking or delayed. The highest conversion frequency was found with four-week-old cotyledonary embryos, while the lowest number of plantlets with two primary leaves was recorded with two-week-old embryos.

Figure 3.3. Twenty-five-day-old sucrose (A) and PEG (B) embryos on black filter paper (Gelman Sciences) placed on solid germination medium. Two days later, germination of sucrose (C) and PEG embryos (D). Note elongated radicle and formation of numerous root hairs. Grids on filter paper were 3 mm in width. Haploid plantlets developed from sucrose (E) and PEG embryos (F) after three weeks on solid hormone-free germination medium.


SUCROSE
PEG
A
B
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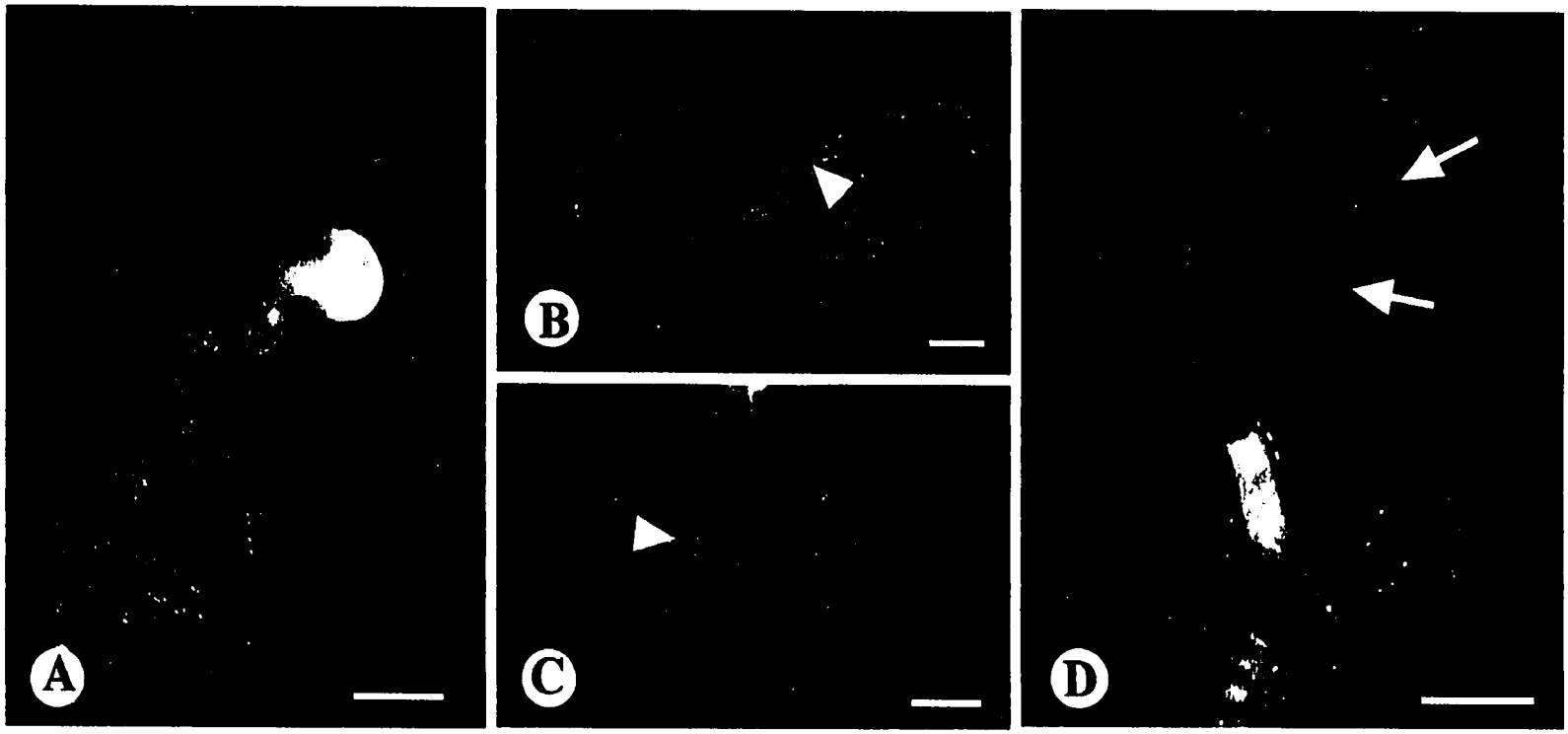
SUCROSE
PEG
E
F

Figure 3.4.A. Formation of secondary embryos on the hypocotyl of a sucrose plantlet. Scale bar = 5 mm. B. Enlargement of hypocotyl region (from A), showing secondary embryos (arrowhead). C. Callus and secondary embryos (arrowheads) on the hypocotyl region in a sucrose plantlet. Scale bars (B and C) = 1 mm. D. Second set of cotyledons on a PEG plantlet (arrows). Scale bar = 5 mm.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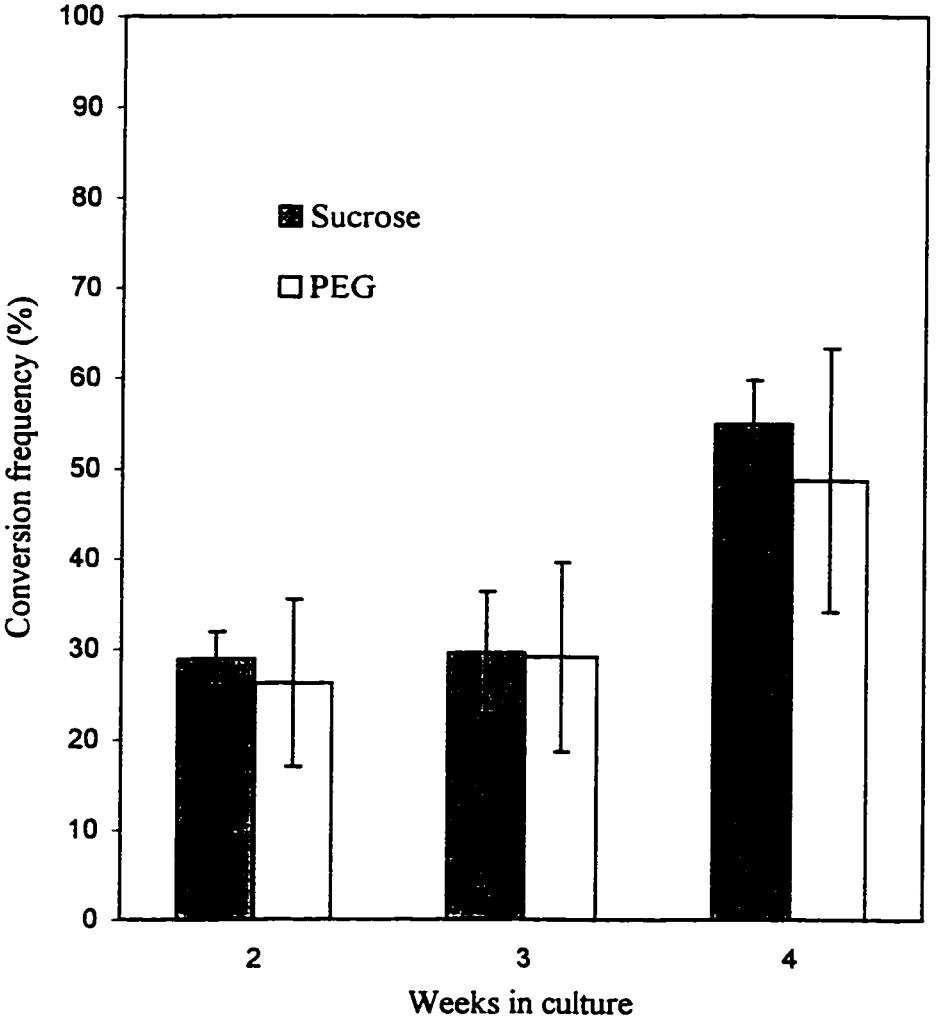

Figure 3.5. Conversion frequency of MD embryos in *Brassica napus* L. at 2, 3 and 4 weeks after microspore isolation. Embryos were transferred to solid germination medium for four weeks. Error bars indicate ± standard deviations.

3.4. Discussion

This is the first complete report of induction of microspore embryogenesis using high molecular weight PEG as an osmoticum, including only a minute quantity of carbohydrate in the medium. Under restricted sugar and water supply, the size of the PEG embryos was reduced when compared to sucrose embryos; however, they were morphologically very similar to immature zygotic embryos dissected from ovules at 14 and 15 DPA (Fig 3.2.E and F). Since the size of MD embryos, rather than their total number was affected on PEG medium, this indicated that a high concentration of PEG indeed provided a proper osmotic environment for induction of microspore embryogenesis in *Brassica napus*. The smaller size of PEG embryos compared to sucrose embryos is likely due to: a) restricted carbon supply, as expected given the minute amount of carbohydrate provided in the medium, and b) continuing water stress conditions. Interestingly, the high viscosity of the PEG solution and restricted water and oxygen supply did not affect early microspore development upon inductive heat shock treatment, which is considered to be a sensitive stage.

In contrast, mannitol, a solute generally considered to penetrate cells passively and very slowly (Cram, 1984), had a negative effect on induction of microspore embryogenesis. A similar effect of mannitol was observed in *Brassica campestris* microspore (A.M.R. Ferrie, personal communication) and anther culture (Hamaoka et al., 1991). Such a negative effect of mannitol could be due to uptake of this solute by microspores in a liquid medium, followed by substantial accumulation of mannitol in the cytoplasm. Uptake and metabolism of mannitol by barley and maize root cells was reported by Cram (1984). Also, uptake of mannitol and its transport to the shoot was reported in *Brassica napus* and *Triticum aestivum* seedlings grown *in vitro* (Lipavská and Vreugdenhil, 1996).

Sucrose has been widely used as an osmoticum as well as a carbon and energy source in anther and microspore culture of a number of species (reviewed by Ferrie et al., 1995). As a

metabolite, sucrose can be absorbed into cell symplasts, readily utilized and/or stored in vacuoles or converted into starch and stored in plastids (Avigad, 1982). Invertases (acid and neutral forms) and sucrose synthase catalyze the breakdown of sucrose molecules. Through an apoplastic route, hydrolysis of sucrose is catalyzed by cell wall-bound acidic invertase (an insoluble fraction) resulting in the hexose monomers, glucose and fructose, which are then taken up into the cytoplasm. Embryos on high sucrose medium were pale green with elongated embryo axes and relatively small, underdeveloped and often abnormal cotyledons (Fig 3.2 and Fig 3.3). These features have already been reported in *Brassica napus* MD embryos (Rahman, 1993; Hause et al., 1994; Yeung et al., 1996). Based on the results of the present experiments, it is clear that a sucrose-abundant environment affects not only the internal cell and tissue structure of MD embryos, as reported by Rahman (1993), but also their external morphology, i.e., size and shape of cotyledons as well as size of the whole embryos (Fig 3.2.D).

Besides these differences, embryo colour as well as the appearance and length of hypocotyls, observed later during plantlet formation, were also strikingly different (Fig 3.3). The differences described could be due to the unbalanced sugar supply which has a direct effect on sugar metabolism, with sucrose and PEG embryos representing two extreme cases. However, it is known that sugars are involved in regulation of plant growth and development through physiological signals, repressing or activating genes involved in many processes (Jang et al., 1997). The mechanism by which plant cells sense sugars and further process this information is largely unknown and only recently sugar signaling pathways and sugar responsive gene expression have been studied extensively in plants (reviewed by Koch, 1996; Jang and Sheen, 1997). Phosphorylation of hexoses by the enzyme hexokinase (HXK) appears to initiate the signaling cascade, since only hexoses that can be phosphorylated by HXK are effective. Thus, HXK was proposed to be a sugar sensor in plants, playing a crucial role in plant response to intracellularly perceived hexose signals (Jang and Sheen, 1997). Using the reverse genetics approach, Jang et al. (1997) have shown that hypocotyl elongation

and cotyledon greening of transgenic *Arabidopsis* seedlings were suppressed by high glucose in the medium, with HXK mediating glucose-regulated gene expression. The authors concluded that sugar negatively regulates hypocotyl elongation, suggesting the possibility of cross-talk between sugar and hormone signal transduction. Assuming that sucrose was converted to its hexose components in the apoplast of sucrose embryos before it became a signal, a hexose signaling mechanism could account for the differences in sucrose and PEG embryos and plantlets described in the present experiments. In order to test this hypothesis, experiments with microspore and embryo culture need to be combined with a molecular genetics approach; otherwise, only a very limited amount of information can be obtained.

In the experiments described here, conversion frequency reflects a capacity of MD embryos to develop root and shoot systems (Stuart and Strickland, 1984), rather than their actual survival rate and development in *ex vitro* environment, since haploid plantlets were not transferred to soil. The term 'germination' is used when referring to the elongation of radicle and root hair formation once MD embryos were placed on solid germination medium. Overall, haploid plantlet formation from MD embryos was relatively low. The highest conversion frequency (>50%) was recorded in four-week-old embryos on both osmotica. This corresponds to the previously reported data on germination rates in MD embryos induced on high sucrose (Kot and Beversdorf, 1990) but contrasts with the data on conversion frequency of MD embryos published by Yeung et al. (1996) who reported the highest germination frequency in two-week-old MD embryos. A possible explanation for higher conversion frequency in older embryos (on hormone-free germination medium) might be due to the difference in hormonal balance between four-week-old MD embryos and younger embryos, since this balance changes over time, as found in *Brassica* microspore embryos cultured on standard high sucrose medium (Mandel, 1992).

Development of extra cotyledons as well as callus and secondary embryo formation on hypocotyls has been reported in precociously germinated zygotic embryos in *B. napus*

(Finkelstein and Crouch, 1984). According to these authors, such phenomena were a result of parallel and concurrent processes specific for both embryogeny and germination, which occurred when immature embryos were exposed to conditions that favored germination. Therefore, they concluded that the capacity of embryos to develop into normal seedlings was acquired gradually during maturation and whenever the maturation processes were not completed, the normal (complete) transition from embryonic to germinative developmental mode was disrupted. In the experiments described in this section, development of MD embryos proceeded in the absence of applied ABA, an important hormonal factor that triggers maturation events, and therefore they were in an "immature" state at the time they were placed on filter paper for germination. Assuming that the features of haploid plantlets described here are analogous to those found in precociously germinated immature zygotic embryos, a proper experimental design which facilitates *in vitro* maturation and subsequent desiccation of MD embryos would result in formation of normal and vigorous plantlets. These experiments have been conducted as described in Section 5.

3.5. Summary

The results of these experiments demonstrate that sucrose as an osmoticum is not essential in microspore embryogenesis of *Brassica napus*; it can be replaced with high molecular weight PEG. The amount of metabolizable carbohydrate required for MD embryo induction and formation appears to be substantially less than reported previously. Morphologically, PEG embryos appeared remarkably similar to immature zygotic embryos dissected from ovules. The formation of haploid plantlets with functional root and shoot apices was relatively poor, with the best results in four-week-old MD embryos on both sucrose and PEG medium. In the method described here, osmotic and nutritional requirements are uncoupled by using the non-carbohydrate polymer PEG 4000 as an osmoticum of choice. This strategy allows proper calculation of the amount of carbohydrates required for the development of MD embryos in each experiment, using parameters such as microspore plating density,

approximate frequency of embryogenesis, average fresh and dry weight of embryos per plate. Such a flexible system permitting manipulation of carbohydrate sources and amounts while the osmotic pressure of the medium remains constant can be customized for a wider range of genotypes. Therefore, successful haploid embryo induction might be achieved for some commercially important species that exhibit recalcitrance in microspore culture using standard protocols.

3.6. Literature Cited

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Section 4. MORPHOLOGICAL STUDY OF MD EMBRYOS AS REVEALED BY SEM AND LIGHT MICROSCOPY

4.1. Introduction

Despite the large body of experimental work on haploid embryogenesis via anther and pollen culture, there has been a limited number of structural studies illustrating the entire time-course of haploid embryo development. Even fewer are comparative structural studies of *in vitro* haploid embryos and zygotic embryos developed within seeds with exceptions in tobacco anther culture (Norreel, 1972) and more recently in *Brassica napus* microspore culture (Hause et al., 1994; Yeung et al., 1996). At the SEM level, a detailed study of early events during microspore embryogenesis in *Brassica* spp was reported by Nitta et al. (1997). In other species, SEM studies were undertaken in pollen embryogenesis of *Hyoscyamus niger* (Dodds and Reynolds, 1980) and somatic embryogenesis in alfalfa (Xu and Bewley, 1992) and conifers (Fowke et al., 1994).

At the onset of embryo development *in ovulo*, both the egg cell and consequently the zygote are structurally polarized cells (Russell, 1993). The first division of the zygote is asymmetrical resulting in two cells, one giving rise to the suspensor and the other to the embryo proper. The structural characteristics of the zygote seem to be linked to the establishment of the apical-basal axis of the embryo (Dodeman et al., 1997). The bipolar axis of the embryo proper, evident at its transition from globular to heart-shape stages, is therefore delineated before the proembryo itself is formed. In contrast, symmetrical division of isolated microspores upon heat shock treatment is frequently observed in *B. napus* (Fan et al., 1988; Zaki and Dickinson, 1990, 1991; Telmer et al., 1993). Alternation in division symmetry, i.e., disruption of structural polarity in isolated microspores, is suggested to be a key event for

their switch from the gametophytic to the sporophytic pathway (Zaki and Dickinson, 1991; Simmonds, 1994; Telmer et al., 1995). In this section, the possibility of a more complex situation at the onset of embryogenesis in the male gametophyte of *B. napus*, which includes both symmetrical division as well as asymmetrical division of microspores and bicellular pollen, is discussed.

Sucrose has been widely used as an osmoticum as well as a carbohydrate source in anther and microspore culture and was considered an essential medium component for the induction of embryogenesis in *Brassica* (Ferrie et al., 1995). However, the internal cell structure of embryos was altered in a high sucrose milieu - abundant starch accumulation was observed in the cells of both MD embryos and zygotic embryos when cultured on high sucrose liquid medium (Rahman, 1993). To avoid this effect of sucrose, a high molecular weight polyethylene glycol (PEG 4000) was used as an alternative osmotic agent in microspore culture of *B. napus*. As observed in Section 3, three-week-old MD embryos produced on PEG appeared remarkably similar to immature zygotic embryos dissected from ovules. The aim of this section is to describe in detail morphological changes during the entire time-course of microspore embryogenesis in *B. napus* induced on PEG and sucrose using SEM and to examine the differences in starch accumulation in sucrose and PEG embryos at the light microscope level. Evidence related to the establishment of polarity in microspore-derived embryos is presented and the possible origin of polarity is discussed.

4.2. Materials and Methods

4.2.1. Growth conditions of donor plants

Donor plants were maintained in a growth chamber as described in Section 3.2.1.

4.2.2. Isolation and culture of microspores

The method is described in Section 3.2.3.

4.2.3. Scanning electron microscopy

Microspores and MD embryos cultured on sucrose medium were collected into 1.5 ml Eppendorf tubes at 6, 9 and 14 days after isolation and fixed sequentially in 1% and 3% glutaraldehyde in 0.025 M phosphate buffer prepared in 10% sucrose. Those cultured on PEG medium were first washed for 3 min in half-strength NLN medium containing 8% mannitol, then fixed sequentially in 1% and 3% glutaraldehyde in 0.05 M phosphate buffer made with 8% mannitol. Samples were further processed as described in Section 2.2.3.

4.2.4. Light microscopy

MD embryos were fixed as described in Section 2.2.3. and dehydrated, infiltrated and embedded in Araldite as described in Section 2.2.4. Semithin sections (1-1.5 μm) were mounted on poly-L-lysine-coated slides (0.1%, w/v) and stained with Sudan B as described by Bronner (1975). To determine the developmental stage of microspores, small samples from each treatment were collected after the first centrifugation in 0.5 ml Eppendorf tubes and stained with aqueous DAPI solution, final concentration 2 μg ml $^{-1}$ in 1% Triton X 100, (Coleman and Goff, 1985). Microspores were gently spun and immediately observed with a

Zeiss Axioplan epifluorescence microscope equipped with HBO 50 W mercury lamp and standard filter set for DAPI.

4.3. Results

4.3.1. *In vitro* development of MD embryos on sucrose and PEG medium

During the first six days in culture, the enlargement of embryogenic microspores and young pollen proceeded in a very similar manner on both osmotica. Thus, after heat shock treatment, a portion of the total population of microspores swelled and further increased in size, due to first cell divisions within the original pollen wall. They eventually reached 35-50 μm in diameter before the final breakdown of the pollen wall, at about day 5 in culture. Three different types of morphological changes were observed during this period (Fig 4.1). In type I (Fig 4.1.A), swollen microspores lost their original tricolpate structure and became spherical, as the germination furrows flattened. The pollen wall stretched uniformly over the entire surface, and expanding ridges of reticulate exine sporadically cracked. In the present study, however, there is little evidence of how the final breakdown of pollen wall occurred, and more importantly, of whether embryogenic development continued beyond the first six days in culture. In another, more common type (type II), enlargement of the microspore caused a split in the pollen wall along all three colpae, with a volume increase localized primarily in these regions, while the reticulate exine stretched and changed less than in type I (Fig 4.1.B). After five days in culture, the growing cell cluster usually broke out along one of the ruptured furrows, while remnants of the pollen wall remained adhering to the side of a cluster (Fig 4.1.C). In this type, embryonic development proceeded and within days 'spheroid' structures were formed with the pollen wall still adhering firmly to their surface (Fig 4.2.A and F). In type III, periodically observed on both osmotica, all three colpae cracked as the growing microspore expanded; however, instead of forming a compact multicellular cluster, only a few enlarged and loosened cells emerged, eventually causing the pollen wall to break down entirely along one of the ruptured furrows (Fig 4.1.D). These cells likely gave rise to non-embryogenic calli (clumps of loosely associated cells) often observed in embryogenic microspore culture (Fig 4.1.F). It is noteworthy that the final breakdown of

the pollen wall appeared to be a critical point during the early growth of embryogenic microspores *in vitro*. A collapse of the enlarging embryogenic microspores upon the breakdown of the original pollen wall was frequently observed on both osmotica (Fig 4.1.E). Once released from the constraints of the pollen wall, these embryogenic structures often had two distinct regions: a globular cluster of cells that divided continuously and gave rise to the whole MD embryo, and a smaller region consisting presumably of one or a very few cells, covered with remnants of pollen wall (Fig 4.2.A and F). The cell(s) enclosed by the pollen wall at first appeared quiescent, and when division occurred, suspensors were formed.

After the first week in culture, differences between sucrose and PEG embryos gradually became more obvious. The embryos enlarged substantially prior to the first evidence of transition to bilateral symmetry. In a strict sense, these structures were not radially symmetrical; in most cases, they were more or less irregular in shape, elongated or oval (Fig 4.2.A and F). Embryos enlarged more rapidly on sucrose medium, where they reached 150-300 μm in length before the first sign of globular-to-heart stage transition, usually occurring about day 8 in culture (Fig 4.2.B). PEG embryos were about 100-150 μm in diameter when the flattened apical region became slightly concave, and emergence of cotyledonary lobes on the oval-shaped embryos was apparent (Fig 4.2.G). Suspensors were often observed at this stage or earlier in both sucrose and PEG embryos. They apparently arose as a result of transverse cell divisions in particular peripheral cell(s) still covered by remnants of pollen wall. Emergence and outgrowth of two cotyledonary lobes and elongation of the embryo characterized the heart stage of embryo development. Sucrose embryos were approximately 300 μm or more in length at this stage (Fig 4.2.C), while PEG embryos were approximately half that size (Fig 4.2.H). At this stage, a range of different shapes and sizes of suspensor was observed, from filamentous uniseriate structures (Fig 4.2.H) to small, multicellular protrusions, irregular in shape (Fig 4.2.B). However, not all MD embryos exhibited these structures. Also, there was variability among experiments; in some, the majority of embryos had suspensors while in others few distinct suspensors were found. The emerging cotyledons

expanded rapidly and the embryo axis continued to elongate, giving rise to early torpedo stage embryos. Sucrose embryos reached a size of about 400 μm or larger, primarily as a result of axis elongation, whereas cotyledons were rather short and undeveloped, their expansion slow and delayed (Fig 4.2.D). PEG embryos at the torpedo stage had enlarged cotyledonary lobes and were about 300-400 μm in length (Fig 4.2.I), similar to zygotic embryos at this stage.

Two-week-old cultures already contained some cotyledonary stage embryos, although the bulk of the embryos were still at the torpedo or heart-shaped stage. Also, globular clumps of non-embryogenic undifferentiated cells were often observed. Early cotyledonary stage embryos were approximately 2-4 mm in length on sucrose medium (Fig 4.2.E) and only 0.7 mm to 2 mm on PEG medium (Fig 4.2.J). Sucrose embryos had rather small, underdeveloped cotyledons and a disproportionately elongated embryonic axis. Very often radicles showed some sloughing of epidermal cells (Fig 4.2.E), which progressed toward the apical region as the embryos became older. In contrast, PEG embryos had two well-developed cotyledons and proportionate embryo axis with a smooth epidermal surface and were remarkably similar to the early cotyledonary embryos developed *in vivo* (Fig 4.2.J cf. Fig 2.3.G). Once placed under light, all MD embryos increased in size and became green. PEG embryos had dark green cotyledons (often widely opened), whereas sucrose embryos had small, pale green cotyledons and very elongated embryo axes.

4.3.2. Pollen wall fragments attached to MD embryos

At about day 9 in culture, suspensors were observed on globular and heart-shaped embryos on both osmotica; however, not all embryos had these appendages. The shapes and sizes varied from long filamentous suspensors (Fig 4.3.C) to small irregular shaped cell clusters (Fig 4.2.B, C, and D). Also, some MD embryos had a rather rudimentary protrusion at the future root pole (Fig 4.3.A and B), or no evidence of a suspensor at all. Fragments of the original pollen wall were consistently observed at the tips of the suspensors (Fig 4.3.A, B, and

C), regardless of their shapes or sizes. In suspensor-less embryos, exine patches were attached to the embryo surface at the future root pole (Fig 4.3.D). Occasionally, small pieces of the pollen wall were also found elsewhere on the embryo surface. Since the reticulate exine was easily detected by SEM, it became evident that most embryos had these patches, primarily at the future root pole region. They were rather conspicuous on smaller embryos (globular, heart and torpedo stages) and less noticeable on large cotyledonary stage embryos, due to their large size compared to the minute size of these fragments. These exine remnants were regularly observed on MD embryos on both osmotica throughout their development.

Figure 4.1.A-F. Representative examples of different morphological changes in microspores of *Brassica napus* L. during the first several days in culture as revealed by SEM. A. Type I: Swollen round microspore with evenly expanding pollen wall and indistinct germination furrows (arrows). Note broken ridges of the reticulate exine (arrowhead). B. Type II: Enlarged microspore with all three colpae ruptured (arrows). C. Globular cluster emerging from one of the furrows (arrow). D. Type III: Three loosely associated non-embryogenic cells breaking out from the pollen wall. E. Collapsed microspore after the breakdown of pollen wall. Scale bars (A-E) = 10 μm. F. Cluster of non-differentiated cells observed after nine days in culture. Scale bar = 50 μm.

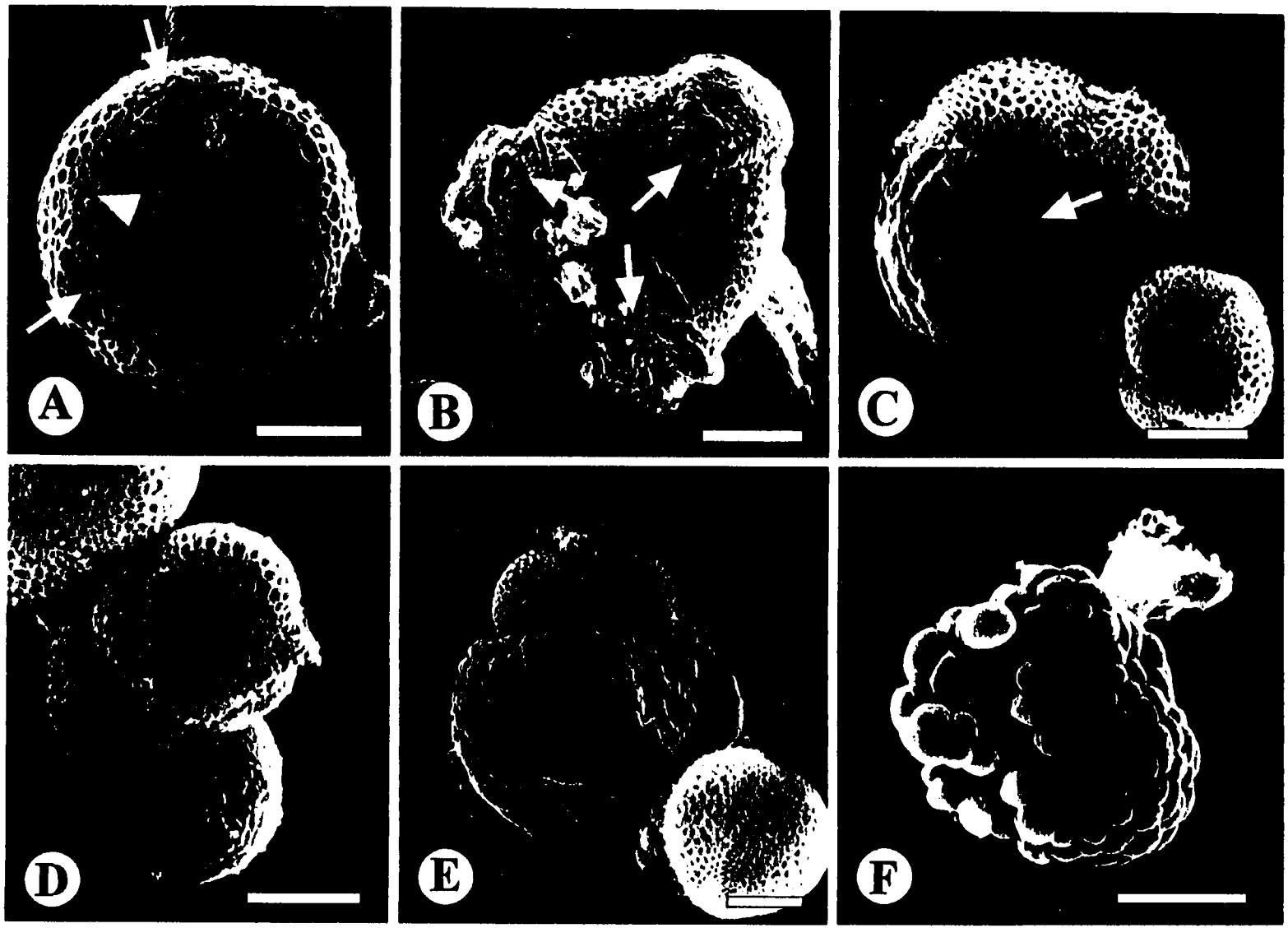
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Figure 4.2. Developmental stages of MD embryos cultured on sucrose (A-E) and PEG (F-J). A. Globular cluster of cells rapidly enlarging upon the breakdown of pollen wall. Note the region with tightly adhering remnants of pollen wall (arrow). Scale bar =10 μm. B. Oblong embryo with flattened terminal end (arrow) and a suspensor on the basal end (arrowhead). C. Heart stage embryo. D. Early torpedo stage embryo. Scale bars (B-D) =100 μm. E. Two-week-old sucrose embryo at early cotyledonary stage with elongated embryo axis (EA) and small cotyledons (CO). Note abrasion of the epidermis near the radicle. Scale bar = 1 mm. F. Globular PEG embryo shortly after breakdown of pollen wall. Scale bar = 10 μm. G. 'Oval-shape' embryo with concave terminal end (arrow) and suspensor protrusion at the opposite end (arrowhead). H. Heart-stage embryo with long filamentous suspensor (SU). I. Early torpedo-stage embryo with multicellular tip of the suspensor (arrowhead). J. Early cotyledonary stage PEG embryo with two cotyledons (CO) and embryo axis (EA). Scale bars (G-J) = 100 μm.

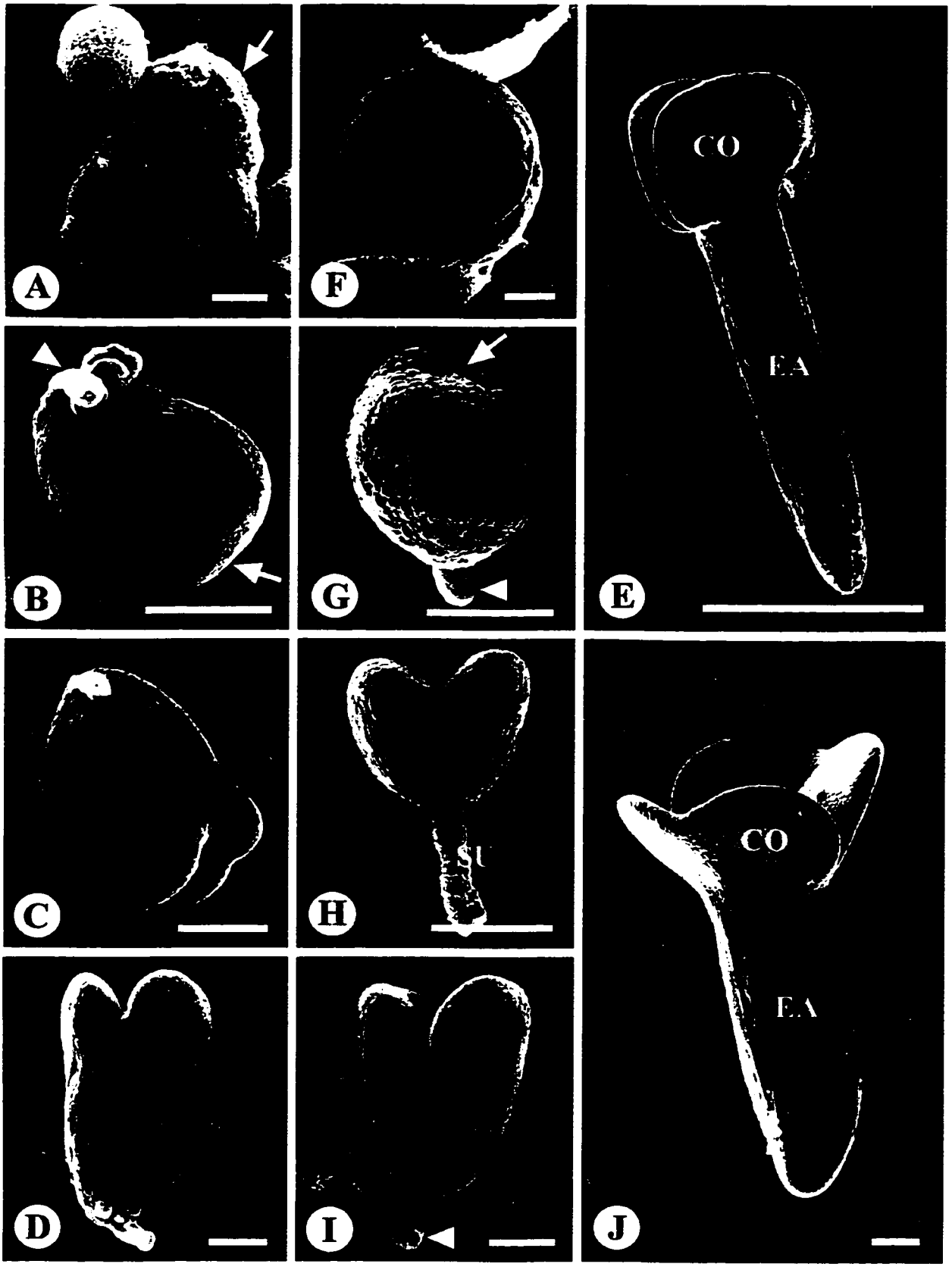
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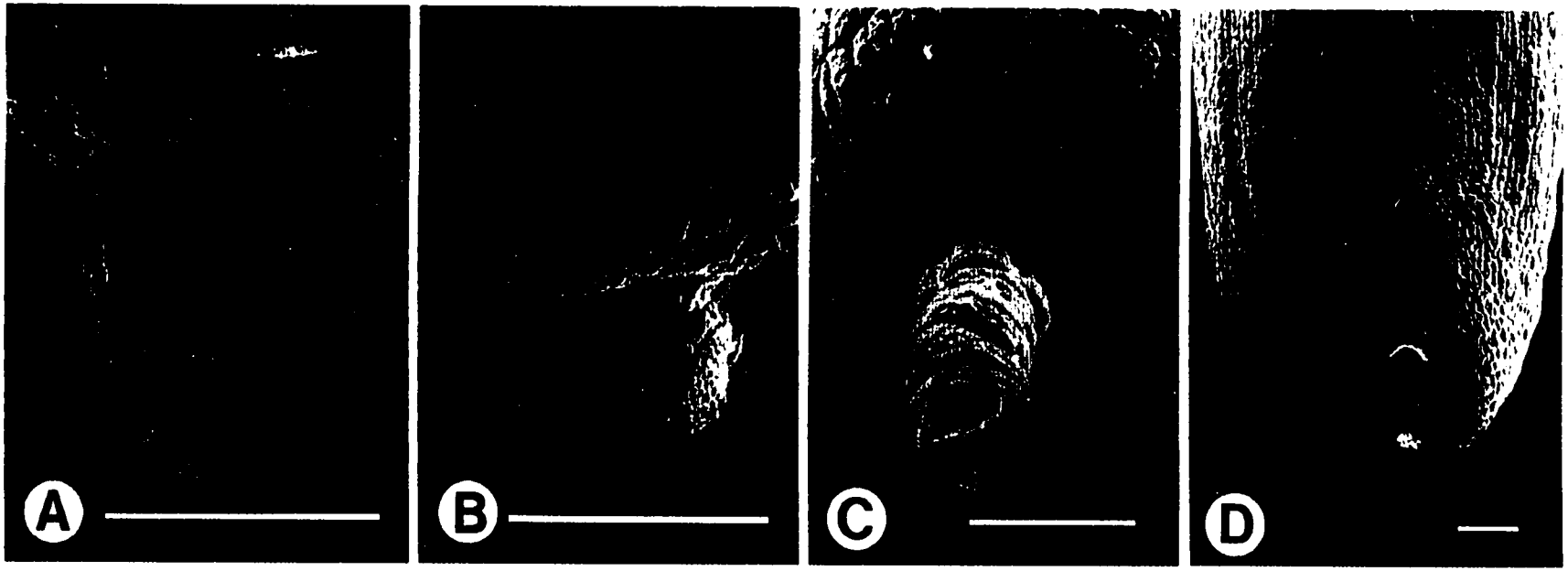
Figure 4.3.A-D. Remnants of original pollen wall adhering to the tips of suspensors (A-C) and to the root pole region (D) in MD embryos at different developmental stages: A. PEG embryo at globular stage. B. Sucrose embryo at early torpedo stage. C. Heart-stage embryo on PEG. D. Two-week-old cotyledonary embryo on PEG. Scale bars = 40 μm.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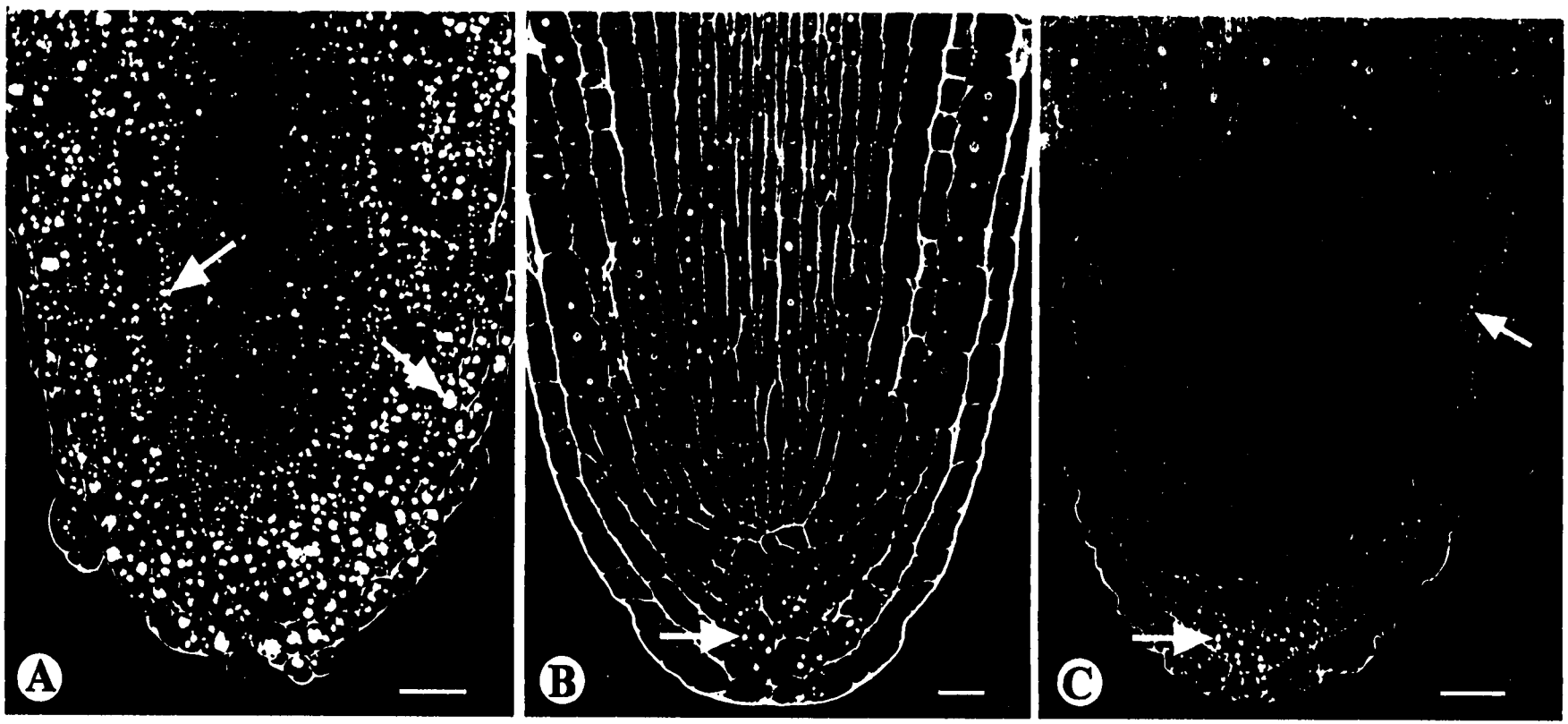
D

4.3.3. Starch and lipid accumulation in sucrose and PEG embryos - histochemical study

Choice of osmoticum had a direct effect on internal structure of MD embryos. Sucrose embryos had large intercellular spaces and literally all cells filled with starch grains, mainly very large aggregated granules which occupied most of the cell volume (Fig 4.4.A). In contrast, PEG embryos were almost completely depleted of starch, except in the columella region and few adjacent cells at the embryonic root pole. These were the only cells with larger starch granules (Fig 4.4.B). Zygotic embryos at 15 DPA had numerous but very small starch grains evenly distributed in the cortex of the embryo axis. Large starch grains were located exclusively in cells of the columella layers and surrounding cells of the root cap (Fig 4.4.C, arrow). PEG embryos contained minute lipid bodies while cells of the sucrose embryos had much larger lipid bodies (stained black with Sudan B).

Figure 4.4. Structure of the root pole of MD and zygotic embryos at early cotyledon stage (Araldite embedding). Note: starch grains remained unstained (arrows) after staining with Sudan B. A. Two week-old sucrose embryo. B. Two week-old PEG embryo. C. Zygotic embryo developed *in vivo* (15 DPA). Note the difference in wall thickness. Scale bars = 20 µm.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4.4. Discussion

4.4.1. Morphology of MD embryos induced on sucrose and PEG-mediated low water potential medium

During the first six days in culture, morphological changes observed while the microspores were still within their original pollen wall were similar on both osmotica. Three developmental patterns were identified but only one (type II) appeared to give rise to MD embryos. In contrast, Nitta et al. (1997) described two types of morphological changes in embryogenic microspores of *Brassica*, both giving rise to MD embryos. The authors suggested that each type may originate from a different developmental stage, i.e., late uninucleate microspore or early bicellular pollen. In both studies, multiple bud experiments were performed, resulting in highly heterologous microspore populations consisting of several developmental stages. Therefore, in the present study predominance of type II may have occurred as a result of a higher percentage of early bicellular pollen switching to the embryogenic pathway upon heat shock treatment.

After the first week in culture, as MD embryos enlarged, differences between sucrose and PEG embryos became apparent. Suspensors, small protrusions at the future basal pole, appeared periodically on both sucrose and PEG embryos, yet PEG embryos often had filamentous uniseriate suspensors which, in some cases, exceeded the length of the embryo itself. Another striking difference between the sucrose and PEG embryos was cotyledon size. In PEG embryos, differentiation of cotyledons proceeded normally, from emerging distal lobes at the heart stage to the large cotyledons of three-week-old embryos. In contrast, small underdeveloped and sometimes aberrant cotyledons were regularly observed on sucrose embryos. Such cotyledon features were documented previously in MD embryos of *B. napus* induced on sucrose medium (Fig 4 and Fig 5 in Hause et al., 1994) and in alfalfa somatic embryos (Xu and Bewley, 1992).

Under limited sugar and water supply in PEG medium, embryo size was reduced; however, morphologically, these embryos appeared superior to those cultured on sucrose, as illustrated in Section 3 (see also Ilić-Grubor et al., 1998). When sucrose was included in NLN-PEG medium at much higher levels, e.g., 4% and 6.25% (data not shown) and 13% in the present study, embryo morphology was markedly altered. Therefore, even in the PEG-mediated water restricted environment, the presence of higher levels of sucrose in the medium had a profound effect on the morphology of MD embryos. The peculiar internal structure of sucrose embryos, with large and abundant starch grains and wide intercellular spaces, as illustrated in Fig 4.4.A, was reported previously in *B. napus*. When both zygotic and MD embryos were cultured on high sucrose medium, the internal cell structure of the embryos was altered, as substantial starch accumulation took place (Rahman, 1993). However, when the amount of sucrose in the medium was reduced to a minute quantity, as performed in the present experiments, accumulation of starch in MD embryos was reduced dramatically (Fig 4.4.B). Since the presence of sucrose in the medium directly affected starch accumulation in MD embryos, this process could be controlled by manipulating the sucrose levels *in vitro*.

4.4.2. Establishment of polarity in MD embryos

The SEM provided an unique opportunity for visualization of surface-associated changes occurring very early in microspore embryogenesis. The occasional formation of suspensors, observed in MD embryos during the first 7-10 days in culture, has been previously reported (Pechan et al., 1991; Hause et al., 1994; Yeung et al., 1996). These suspensors were characterized as a structural manifestation of polarity established within the microspore embryo (Yeung et al., 1996). Hause et al. (1994) observed a polar distribution of starch during the pollen wall rupture in *B. napus* microspore embryogenesis, suggesting that polarity of MD embryos was induced at this particular stage by rupture of the pollen wall. Although in the present study, the first visible sign of polarity in MD embryos was indeed apparent upon breakdown of the pollen wall, in fact, polarity of MD embryos seemed to be inherited from

the young gametophyte which became structurally polarized as the nucleus moved laterally toward the pollen wall in the late uninucleate microspore. The SEM observations and light microscopy using DAPI revealed some important details which support this view. When suspensors were formed, the patches of reticulate exine were always found at their tips. In the absence of suspensors, these remnants of the original pollen wall adhered to the surface of the future root pole of MD embryos. Immediately after the first mitotic division, the lens-shaped generative cell of the early bicellular pollen is in close contact with the intine (Zaki and Dickinson, 1991; Telmer et al., 1993). This stage, as well as the late unicellular microspore stage, constitutes a developmental window within which the young pollen is potentially embryogenic (Fan et al., 1988; Pechan and Keller, 1988; Telmer et al., 1992; Hause et al., 1993). The pollen wall fragments detected on MD embryos could in fact be remnants of the original pollen wall that were associated with the generative cell via the intine at the early bicellular stage. At this stage, the lens-shaped generative cell is positioned laterally, usually opposite one of the germination furrows. If this cell remained quiescent upon heat shock treatment, as described by Hause et al. (1993) while the vegetative cell divided producing a multicellular cluster, the mechanical pressure built up within the pollen wall would cause the furrow opposite the generative cell to break, releasing the multicellular cluster, while the generative cell remained covered with fragments of pollen wall. Whether the generative cell remained permanently quiescent or started dividing later, possibly giving rise to the suspensor (or contributing to embryo formation some other way), its lateral position seems to delineate the future axis of MD embryos. In the experiments described here this appeared to be a pattern, rather than just a random event.

In this study, a heterologous population consisting mostly of late uninucleate microspores and bicellular pollen was repeatedly observed when multiple bud experiments were conducted. While early bicellular pollen undergoes embryogenic development as described above, late uninucleate microspores may proceed through embryogenesis via the following routes:

i.) late uninucleate microspores may first undergo symmetrical division forming two

equal cells. Usually only one daughter cell continues to divide, giving rise to the embryo (Fan et al., 1988). In this case, the lateral position of the nucleus before the division might be sufficient for the later expression of polarity in MD embryos. Therefore, in a strict sense, this may not be considered a true symmetrical division, despite the morphological symmetry, since the resulting two daughter cells have different fates (Horvitz and Herskowitz, 1992). True symmetrical division seems to occur only when callus formation is initiated. Interestingly, in the published work of Zaki and Dickinson in 1990 (Fig 11) and 1991 (Fig 7 and Fig 8), the shape of cells after the first symmetrical division corresponds to that during early callus formation shown here in Fig 4.1.D,

ii.) late uninucleate microspores may first divide asymmetrically forming the vegetative cell and the generative cell, immediately followed by subsequent division of the vegetative cell which will give rise to most of the MD embryo.

Based on experimental work on haploid embryogenesis via anther and microspore/pollen cultures in other angiosperm species, e.g., *Datura* (Dunwell and Sunderland, 1976), barley (Sunderland et al., 1979) and wheat anther culture (Reynolds, 1993), and tobacco pollen culture (Touraev et al., 1996), it is reasonable to expect a more diverse situation during *in vitro* induction of embryogenic development from the male gametophyte in *B. napus*.

Nonetheless, at present, there is some evidence that polarity of MD embryos might be established as early as microspore and young pollen stages, being analogous to polarity of the zygote, and this idea needs to be investigated further with a multidisciplinary approach.

4.5. Summary

The results presented in this section strongly support the view that sucrose in high concentrations indeed has a morphogenetic effect on MD embryos in *B. napus*. After heat

shock, a small portion of the total microspore and pollen population switched to the embryogenic route. Initially, enlargement of embryogenic microspores and young pollen, while still enclosed within their original walls, proceeded in a very similar manner on both osmotica. After the first week in culture, the difference between sucrose and PEG embryos was distinct as revealed by SEM and light microscopy. Their size differed considerably as well as the size and shape of cotyledons throughout heart, torpedo and early cotyledonary stages. At the light microscopy level, the internal structure of sucrose and PEG embryos differed profoundly. Large starch grains were observed in cells of sucrose embryos at the early cotyledonary stage, but were completely absent in PEG embryos except in the columella region. Fragments of the original pollen wall were regularly observed at the root pole region and at the tips of suspensors in MD embryos throughout their development. This suggests that polarity in MD embryos might originate from structurally polarized late uninucleate microspores and early bicellular pollen.

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Section 5. MATURATION AND DESICCATION TOLERANCE OF MD EMBRYOS

5.1. Introduction

Physical, nutritional, hormonal and osmotic factors are involved in the continuation of seed development during maturation. Among these factors, ABA and restricted water uptake have been most widely studied. Abscisic acid has been implicated in the control of many events during embryogenesis including inhibition of precocious germination, induction of reserve protein synthesis and Lea (Late-embryogenesis-abundant) protein synthesis, induction of desiccation tolerance as well as inhibition of growth and maintenance of dormancy (Black, 1991). Seed development is terminated by maturation drying, with a gradual loss of water and acquisition of desiccation tolerance. During this final stage, the storage product synthesis ceases, the embryo prepares for desiccation, gradually becomes desiccated and enters into a metabolically inactive or quiescent state (Kermode, 1990). In most angiosperm species, seeds retain their viability after desiccation; this is a final phase of maturation for "orthodox" seeds (Bewley and Black, 1994).

Zygotic embryos developed *in vivo* accumulate high levels of ABA during mid-and late stages of embryo development (Quatrano, 1986). Rapeseed MD embryos had an increased level of ABA at the torpedo stage (see Fig 4-1, Hays, 1996); yet the levels of ABA in MD embryos were lower than in their zygotic counterparts throughout development. Common events associated with embryo maturation *in ovulo* did not occur in *B. napus* MD embryos cultured on standard high sucrose medium (Yeung et al., 1996). Instead, torpedo and cotyledonary MD embryos germinated readily when placed on germination medium (Huang et al., 1991). The conversion frequency of these microspore embryos was relatively poor; anomalous plantlets were often formed, and vigor of plantlets was also poor. Despite the presence of endogenous ABA in MD embryos, exogenous ABA was required for induction of desiccation tolerance in *Brassica* MD embryos (Eikenberry et al., 1991; Brown et al., 1993). Recent

attempts to improve plantlet conversion have been focused on induction of desiccation tolerance in MD embryos in *Brassica* by thermal stress (Anandarajah et al., 1991), cold treatment (Kott and Beversdorf, 1990), ABA treatment (Seneratna et al., 1991; Takahata et al., 1993; Brown et al., 1993) or a combination of ABA and high osmoticum (Wakui et al., 1994). In experiments with alfalfa somatic embryos, desiccation tolerance was improved when elevated levels of sucrose were combined with ABA (Anandarajah and McKersie, 1990a), and with ABA and heat shock (Anandarajah and McKersie, 1990b).

Despite the physiological differences between cultured MD embryos and zygotic embryos surrounded by maternal tissue, their developmental programs during early embryogeny appear very similar; therefore, MD embryos could have the capacity to proceed through the final stages of embryo development (maturation and desiccation) when given the proper environmental stimuli.

The previous two sections have dealt with induction and embryo formation, i.e., early events in microspore embryogenesis. The focus of this section is further manipulation of the culture conditions, in particular ABA and osmoticum, for MD embryos at torpedo or early cotyledonary stages. The experimental design was established to mimic (to a limited extent) the natural *in ovulo* conditions during maturation and desiccation of zygotic embryos. Onset of precocious germination of MD embryos was monitored as well as *in vitro* conversion frequency of desiccated embryos. In the final stage of this section the *ex vitro* acclimatization of haploid plantlets and their subsequent growth in soil were observed.

5.2. Materials and Methods

5.2.1. *In vitro* maturation of MD embryos with applied ABA in a sucrose- and PEG-mediated highly negative osmotic potential environment

A 10 M (±)- *cis,trans*-abscisic acid (Sigma, A-1049) stock solution was made in 75% ethanol and kept in a sealed container in the dark at 4°C. Fresh sterile 200 mM ABA working

solutions were prepared in ½ NLN medium containing either 13% sucrose or 25% PEG. During the third week after microspore isolation, aliquots of 200 mM ABA were added to Petri dishes to obtain the following concentrations of ABA: 0, 10 and 50 µM. The Petri dishes were immediately placed in the dark for the next three days. Then, MD embryos were sieved through Nitex nylon mesh (630 µm pore size for sucrose embryos and 355 µm for PEG embryos), briefly washed in B5 washing medium (with 13% sucrose) and transferred to fresh hormone free ½ NLN medium containing either 18% sucrose or 25% PEG. The PEG medium contained 0.5% sucrose. Plates were placed in the culture room with a 16/8 hour photoperiod and 2000 lux illumination for the next 7 days.

5.2.2. Desiccation of MD embryos in a low humidity atmosphere using improvised desiccation units

The MD embryos previously treated with ABA were briefly washed in washing medium containing ABA (in concentrations as before) immediately before desiccation. They were desiccated on Whatman filter discs placed in 100 x 15 mm Petri dishes. Improvised, hand-made desiccation units, designed by S.M. Attree, consisted of (a) two food containers (2.4 liters), (b) small air pump and (c) Masterflex® silicone tubing (No. 66400-16) with attached Millipore air filters (0.2 µm). Desiccation units were kept in incubators at 10°C. To facilitate slow desiccation, sterile air was pumped through closed containers containing unsealed Petri dishes during a ten-day-period. For fresh and dry weight measurements, small samples (approximately 0.1 g fresh weight) from each treatment were placed into Petri dishes (30 x 10 mm, Falcon 1008), and fresh weight was measured before and after desiccation. Dry weight was measured after two-days in the oven at 95°C.

5.2.3. *In vitro* conversion frequency of rehydrated embryos

For gradual rehydration of MD embryos, filter papers with dry embryos were transferred to sterile Petri dishes containing wet filter discs, previously immersed in ½ NLN medium containing no sucrose or PEG. A day later, germinating MD embryos were transferred to a

solid hormone-free NLN medium containing 1% sucrose and 0.75% agar. There were three containers for each treatment and each container held 50 embryos. Conversion frequency was scored as described in Section 3.2.4.

5.2.4. Direct transfer to soil and *ex vitro* conversion frequency of haploid plantlets

Germinating MD embryos were picked out from wet filter discs in Petri dishes 24 hours after rehydration and transferred to sterile filter discs placed on a solid germination medium. After ten days, vigorous plantlets from both sucrose and PEG treatments were washed in water and planted to soil in Cell Paks placed into flat trays (72 cells per tray) and covered with clear humidity lids. A total of 102 plantlets, developed from embryos initially treated with 50 μM ABA during maturation, were planted for each of the two treatments. Plantlets were watered every day and maintained in the growth chamber at 22°C (day) and 16°C (night) with 16 hours illumination and light intensity of 2000 lux. The clear humidity lids were removed after three weeks. *Ex vitro* conversion frequency of haploid plantlets was assessed four weeks after planting in soil as the number of plantlets with at least two pairs of true leaves divided by the total number of plantlets transferred to soil in each group.

5.3. Results

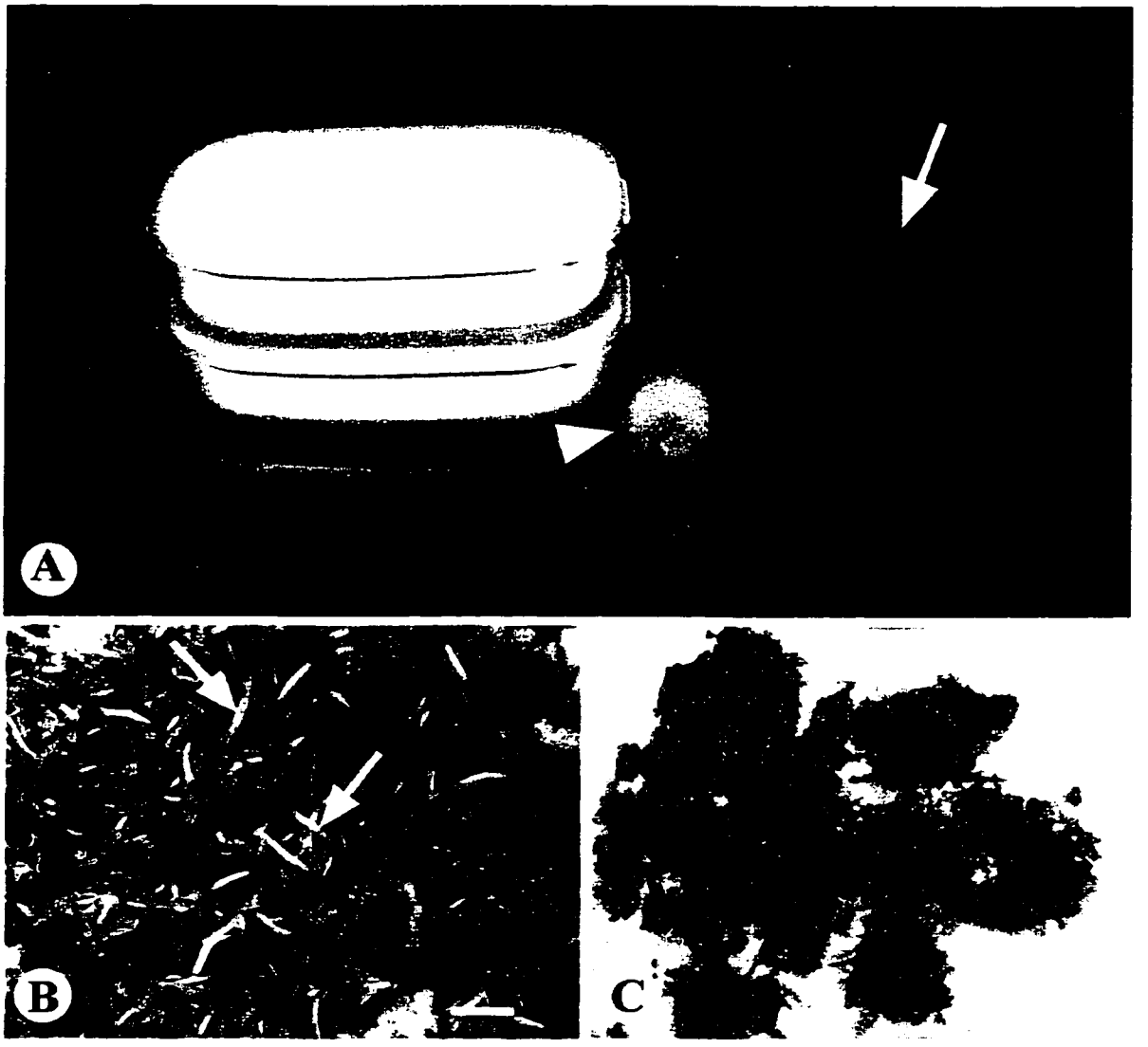
5.3.1. The effect of ABA and high osmoticum on precocious germination of MD embryos

The effect of applied ABA and high osmoticum on suppression of precocious germination and acquisition of desiccation tolerance in MD embryos, cultured under conditions that would promote their maturation, was monitored. In the absence of rapid and measurable methods for assessment of precocious germination in MD embryos, visual observation of elongated embryo axes and radicles was used instead. In an attempt to mimic some of the conditions during embryo maturation *in vivo*, the maturation period was divided into two stages: first, exogenous ABA was applied for three days, followed by transfer of embryos to fresh hormone free medium with high osmoticum. Once sucrose embryos were transferred to fresh

½ NLN medium with 18% sucrose, without previous ABA treatment, the embryos germinated precociously as indicated by rapid elongation of the embryo axes occurred within a few days. Moreover, sucrose embryos which floated on the surface of the liquid medium even developed root hairs. Visible elongation of embryo axes also occurred in sucrose embryos after treatment with 10 μM ABA. Those treated with 50 μM ABA rapidly changed color to yellow, while distinct axis elongation was observed only in a very few embryos. In the absence of exogenous ABA, PEG embryos which floated on the surface of the liquid medium had elongated axes with characteristic yellowish radicles, but no root hairs were formed. Those previously treated with ABA did not exhibit visible axis elongation in the liquid culture. Instead, their colour rapidly changed to yellow shortly after ABA was added in liquid cultures, and they remained yellow even after transfer to ABA free medium and exposure to light. Thus, although signs of precocious germination occurred earlier and were more pronounced in embryos on sucrose than on PEG, neither osmoticum prevented precocious germination of MD embryos in the absence of applied ABA.

Moisture content of desiccated MD embryos varied from treatment to treatment and depended on the duration of desiccation period as well as on temperature conditions during desiccation. After a ten-day-desiccation in an improvised desiccation unit (Fig 5.1.A) at 10 °C, the moisture content of dried embryos (Fig 5.1.B and C) was approximately 10 to 16%. The moisture content of MD embryos before desiccation was 59.12% (±2.97) for sucrose embryos and 62.51% (±4.10) for PEG embryos. Thus, the embryos were dried to approximately 1/5 of their original moisture content.

Figure 5.1. A. An improvised desiccation unit consisting of two food containers (280 mm in length), air pump (arrow) and silicone tubing with attached Millipore air filters (arrowhead). B. Desiccated sucrose embryos (10 μM ABA during maturation) on Whatman filter paper. Note the large size of the embryos and a number of very elongated embryo axes (arrows) C. Desiccated PEG embryos (10 μM ABA during maturation). Scale bar = 5 mm.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5.3.2. *In vitro* plantlet conversion

Upon slow rehydration, embryos not treated with ABA during maturation did not survive desiccation (Fig 5.2). Occasionally, a small number of embryos developed roots (arrows), but rarely turned green and formed shoots. Embryos treated with ABA survived in high numbers, with the highest conversion frequency (over 90%) occurring in embryos treated with 50 μM ABA in both osmotica (Fig 5.3).

5.3.3. *Ex vitro* plantlet conversion

In the *ex vitro* plantlet conversion experiment, distinct morphological differences between sucrose and PEG plantlets were observed soon after their rehydration on filter discs. Sucrose plantlets had vigorous roots, often ten times the length of the shoot system; however, hypocotyls were often thick and short and cotyledons remained closed and small (Fig 5.4.A). PEG plantlets had a normal root system, which apparently was less vigorous than that of sucrose plantlets. The expanded green cotyledons were widely opened and the first pair of true leaves appeared within ten days in most plantlets (Fig 5.4.B). Once transferred from filter discs to soil, the majority of haploid plantlets survived during the critical first weeks under high humidity growth conditions (Fig 5.4.C). However, no expansion of cotyledons or elongation of hypocotyls was observed in sucrose plantlets and very few had the first pair of leaves. In contrast, most of the PEG plantlets developed the first leaves after one week *ex vitro*. Once the clear humidity lids were removed (end of the third week) some of the haploid plantlets from both groups died due to the sudden decrease in relative humidity. The final *ex vitro* conversion frequency, scored a month after planting, was 67.7 % for sucrose plantlets and 86.3% for PEG plantlets (Table 5.1).

Also, a difference in plantlet vigor between these two groups after a month on soil was apparent. PEG plantlets had at least three to four pairs of leaves and were much larger than sucrose plantlets (Fig 5.4.D). Sucrose plantlets remained small during this period and formation of new leaves was delayed. Also, formation of multiple shoots was frequently

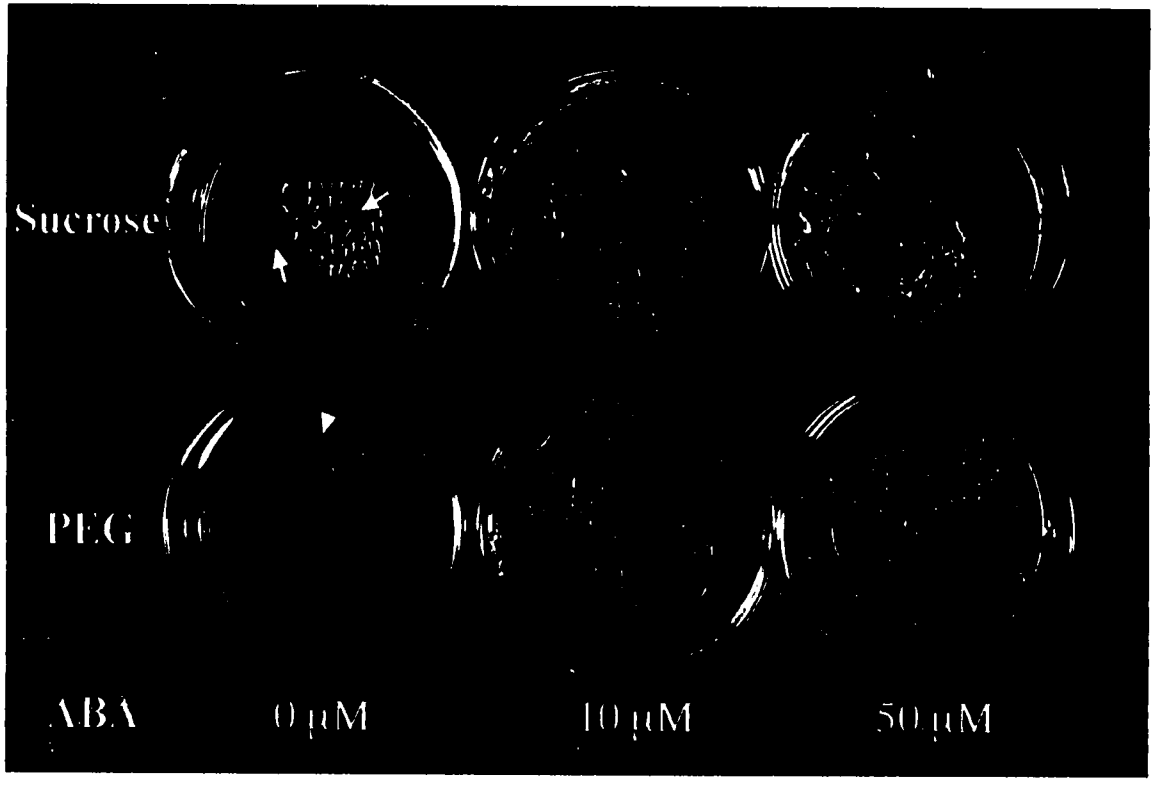
observed in sucrose plantlets.

Table 5.1. *Ex vitro* acclimatization and growth of MD plantlets in soil

Treatment	Total number of plantlets planted	No. of plantlets after 4 weeks*
Sucrose	102	69 (67.65%)
PEG	102	88 (86.27%)

*Plantlets with normal root system and at least two pairs of true leaves.

Figure 5.2. *In vitro* plantlet conversion from desiccated MD embryos 14 days after rehydration and culture on solid hormone-free germination medium in Pyrex containers. Top row, sucrose embryos; bottom row, PEG embryos. The embryos were treated previously with 0, 10 and 50 μM ABA during maturation and shortly before desiccation. Note: Embryos not treated with ABA did not survive desiccation. Occasionally, roots were formed (arrows) and a few embryos turned green (arrowhead) but normal shoot formation did not occur.


Sucrose
PEG
ABA
0 μM
10 μM
50 μM

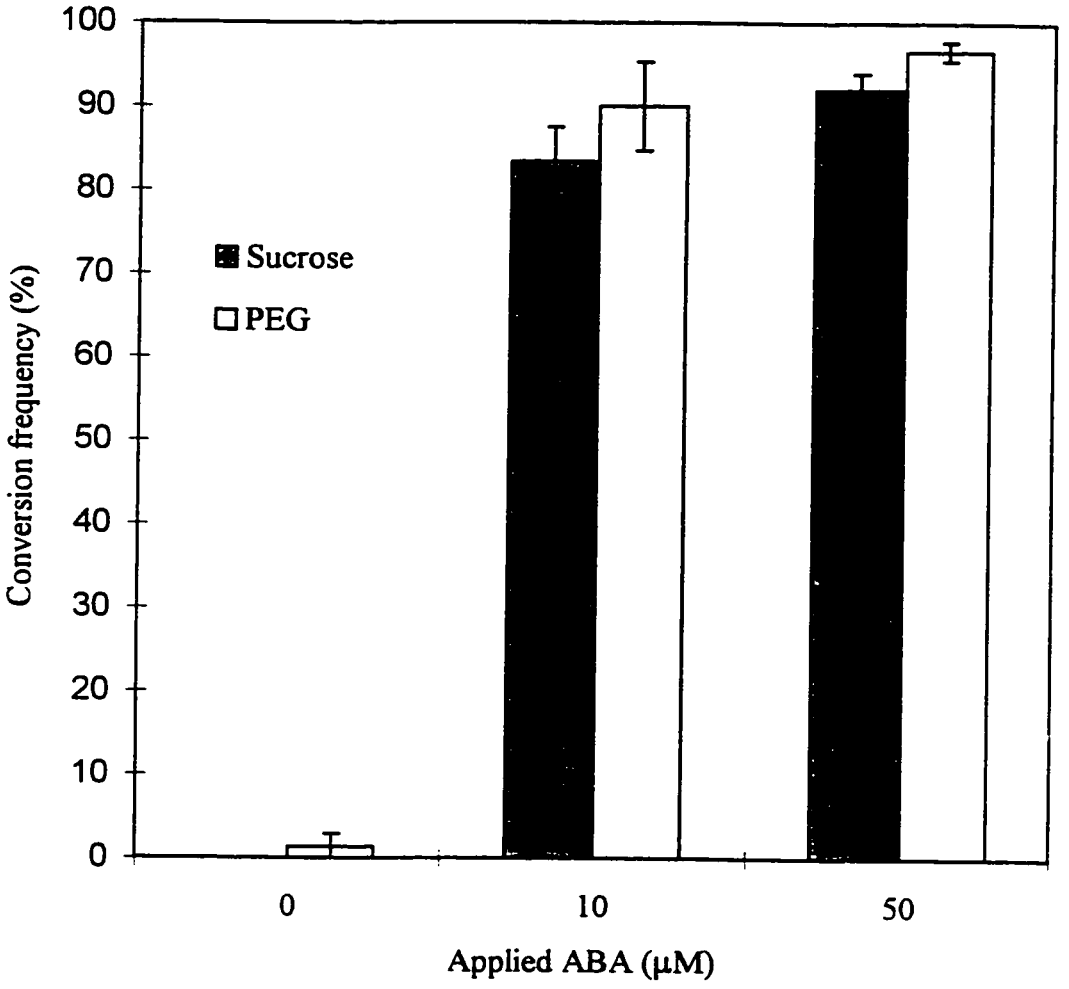
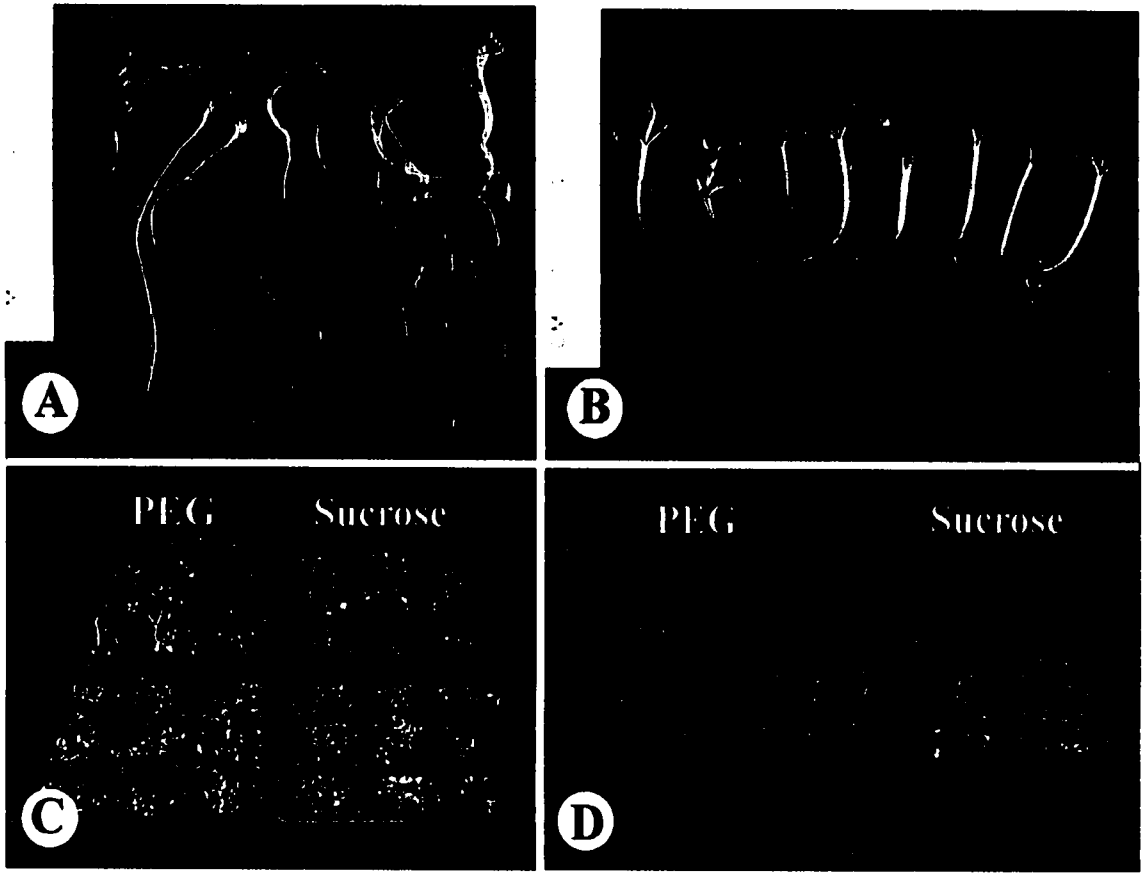


Figure 5.3. *In vitro* conversion frequency of MD embryos after desiccation and rehydration. Rehydrated MD embryos were transferred to solid hormone-free medium and conversion frequency was scored after four weeks.

Figure 5.4.A. Sucrose plantlets developed on Gelman filter discs (ten days after rehydration). B. PEG plantlets derived from rehydrated MD embryos of the same age. C. The same sets of PEG and sucrose plantlets (from A and B) transferred directly to soil after a ten-day-germination period on filter discs. The plantlets were seven days old and were kept under high humidity growth conditions. Left, PEG plantlets; right, sucrose plantlets. Both groups were treated with 50 μM ABA during maturation. D. Haploid plantlets in the growth chamber after one month. Clear humidity lids were removed after three weeks. Left, plantlets from PEG embryos; right, plantlets from sucrose embryos.


A
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PEG
Sucrose
D

5.4. Discussion

5.4.1. Precocious germination of MD embryos

Precocious germination of sucrose embryos after a short treatment with 10 μM ABA during maturation (in 18% sucrose) indicated that even in the high sucrose environment germination processes were triggered. In Petri plates with high embryo density the consumption of sucrose from the liquid medium created a less negative osmotic potential (data not shown). This change in osmotic potential coupled with the abundance of an energy source provided favorable conditions for triggering precocious germination of embryos. In contrast, PEG embryos treated with ABA did not show signs of precocious germination (embryo axes did not elongate) suggesting that they continued their embryonic development on high PEG medium after application of ABA. Therefore, the type of osmoticum used to create high osmotic conditions seems to be important for maintaining embryos in the embryonic developmental mode. By using sucrose as a metabolizable osmoticum, a constant and stable osmotic potential of the medium cannot be maintained and once germination is triggered, an available energy source in the carbohydrate rich medium further facilitates germination of MD embryos.

Nevertheless, in the absence of applied ABA, neither sucrose nor PEG can prevent precocious germination, suggesting an important regulatory role of ABA in suppression of precocious germination. This finding is in agreement with the study of exogenous ABA in zygotic embryo culture in *Brassica* (Finkelstein et al., 1985) and also with several studies of viviparous mutants in maize (Robichaud et al., 1980) and *Arabidopsis* (Koorneef et al., 1984). Why does 10μM ABA in combination with a non-metabolizable osmoticum, such as PEG, efficiently suppress precocious germination of MD embryos, while the same concentration of ABA with metabolizable osmoticum (sucrose) does not? Garciarrubio et al. (1997) showed recently that ABA (30 μM) normally prevents germination of mature seeds in *Arabidopsis*; however, when sucrose and amino acids were added to the medium, this concentration of ABA was ineffective. They concluded that ABA inhibited germination by limiting the availability of

energy and nutrients to seeds. This might also be the case in the experiments described in this section. Since the liquid NLN culture medium used in these experiments has a rich amino acid pool (Gland et al., 1988), once ABA was removed by washing, the high amino acid level in addition to the abundance of sucrose may have been sufficient to promote precocious germination of MD embryos over a seven-day-period.

5.4.2. Desiccation tolerance and plantlet conversion

MD embryos reached the desiccation tolerant state when ABA was added to the medium since only embryos previously treated with ABA survived desiccation. Seneratna et al. (1991) reported more than 50% plantlet conversion from desiccated MD embryos in oilseed rape when 50 μM ABA was applied before desiccation. In their experiments, ABA was supplied in the solid medium, after which desiccation was performed in a series of desiccators with controlled relative humidity. Takahata et al. (1992) reported over 50% plant conversion of desiccated MD embryos in rapeseed, previously treated with 100 μM ABA for 7 days. Furthermore, a very high survival rate (>90%) of desiccated MD embryos in *Brassica* was reported for large cotyledonary embryos treated with 100 μM ABA (Brown et al., 1993) but conversion frequency was not reported. In carrot somatic embryos, application of 38 μM ABA reduced respiration and sugar metabolism and induced complete desiccation tolerance (Tetterloo et al., 1995).

The extremely high conversion frequencies obtained in the experiments described in the present study might be due to a number of important factors, each being crucial for the final outcome. These include proper timing for ABA application during maturation, gradual desiccation and slow rehydration before transfer to a solid medium. If any of these steps were not performed correctly and at the right time, the conversion frequency of MD embryos would have been seriously affected. For instance, rapid rehydration of dry embryos on oversoaked filter papers resulted in death of MD embryos; in other experiments where extra moist filter papers were used, MD embryos survived, but severely hyperhydrated haploid plantlets were developed on solid medium (data not shown). Such abnormal plant material

cannot be used for *ex vitro* acclimatization (Ziv, 1986).

5.4.3. *Ex vitro* acclimatization of haploid plantlets

The morphological differences between sucrose and PEG plantlets which developed directly on filter discs seem to be a result of different levels of stored starch (see Section 4) as well as their *in vitro* growing conditions. Sucrose plantlets had poor and delayed growth of the shoot system and yet long and vigorous roots. The growth strategy of these plantlets appeared to be oriented toward the search for mineral nutrients, while cotyledon expansion and shoot growth was postponed as the availability of energy and carbon source was not limited. In contrast, the growth strategy of PEG plantlets was directed toward leaf and shoot formation as the transition to autotrophic growth was crucial for plantlet survival in the absence of stored starch. In sucrose plantlets, catabolic consumption of sugars continued and therefore transition to autotrophic growth was delayed. In PEG plantlets, however, in the absence of accumulated starch that could be mobilized during acclimatization, plantlet survival relied on production of its own assimilates. These differences resulted in very high PEG plantlet survival during the first month on soil as well as distinct vigor of PEG plantlets.

Direct transfer of germinated plantlets to soil had several advantages over traditional *in vitro* plantlet formation on solid medium. Since plantlets were formed directly on filter discs, and were never in direct contact with agar, the initial loss of plantlets due to contamination in soil was minimized. Also, residues of sucrose from the medium were removed during the washing of plantlets before planting to soil. Hyperhydration (or vitrification) of plantlets, which often occurs in culture systems as a result of high humidity in the head space of the containers as well as several other parameters of culture conditions, such as the medium composition and explants manipulation (Debergh et al., 1992), was also avoided by direct transfer of plantlets to soil.

The methods for maturation and desiccation of MD embryos described in this section combined with a procedure for chromosome doubling should allow direct planting of

rehydrated MD embryos in soil, bypassing *in vitro* conversion of plantlets. The use of growth chambers with controlled relative humidity, enhanced CO_2 and high photon flux (Kozai, 1991) would further enhance the vigor of PEG plantlets transferred to soil. The significant reduction in time required for production of haploid plantlets (total of 10-12 weeks) and the elimination of high costs associated with germination and growth of MD plantlets in sterile conditions should have important economic implications.

5.5. Summary

The use of applied ABA together with osmoticum suppressed precocious germination and facilitated the acquisition of desiccation tolerance in MD embryos of *B. napus*. Exogenous ABA and desiccation treatment improved the conversion frequency of both sucrose and PEG embryos (see Section 3. Fig 3.5 cf. this Section, Fig 5.3). Use of PEG as a non-metabolizable osmoticum allowed direct transfer of haploid plantlets to soil, eliminating time consuming *in vitro* plantlet conversion on germination medium. Improved acclimatization *ex vitro* and rapid transition to autotrophic growth occurred in PEG plantlets resulting in their enhanced vigor. Application of this method in canola breeding programs should further shorten the time for production of haploid lines to approximately ten to twelve weeks. It is now possible to obtain normal vigorous haploid plantlets in a greenhouse within such a short period.

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Section 6. GENERAL DISCUSSION AND FUTURE PROSPECTS

6.1. General Discussion

The objective of this study was to investigate the development of MD embryos in *Brassica napus*, a model system for studies of microspore embryogenesis, and to compare the development of MD embryos with zygotic embryos developed within seeds. A non-conventional, novel approach was undertaken, based on the idea that sucrose, when provided in high concentrations in the medium, likely has an effect on morphological features of MD embryos and their subsequent conversion into haploid plantlets. To test this idea, the amount of sucrose available to embryogenic microspores and developing MD embryos was lowered to residual amounts, while the required negative osmotic potential of the medium was maintained by a high concentration of PEG. Using this approach, a novel method for induction and culture of MD embryos was designed, and MD embryos developed on a low sucrose medium were studied in detail, from the induction stage through embryo maturation and desiccation *in vitro*, to the formation of haploid plantlets and their *ex vitro* acclimatization.

The main question as to how MD embryos survived for four weeks on an extremely low level of carbohydrate in the NLN-PEG medium (approx. 0.1%) is yet to be answered. There are several possible sources for essential carbon and energy supply for embryogenic microspores in this carbohydrate restrictive system. Proplastids, with some starch accumulated, were observed during normal gametophytic development of microspores within locules of anthers (Telmer et al., 1993), and therefore mobilization of plastid reserves could have substituted for the shortage of carbohydrate supply in the medium. Microspores were washed for approximately an hour in a sucrose-rich washing medium during the isolation procedure, and likely absorbed some sucrose from the liquid medium during that period. It is also possible

that MD embryos might have utilized at least some carbon from the PEG molecules. Commercially available high molecular weight PEG (Fluka Chemika) usually contains a small percentage of low molecular weight PEG which could penetrate cell walls. Alternatively, some high molecular weight PEG molecules might have crossed the cell wall via the few large accessible pores, as suggested by Carpita et al. (1979).

One of the most striking events during embryo formation on low sucrose medium with PEG was the rapid and distinct change in embryo colour once they were exposed to light. The rapid chlorophyll formation, as evidenced by the dark green colour of PEG embryos, indicated possible photosynthetic activity. In Petri plates sealed with parafilm, CO_2 (together with the other gases), could have been released by the cells into the head space of the Petri plates. Measurement of the CO_2 and O_2 levels inside the plates may give some indication about photosynthetic activity of green MD embryos.

Results of this study have demonstrated that metabolizable carbohydrate requirements for induction and formation of MD embryos were substantially less than previously reported for *Brassica* microspore culture (see Ferrie et al., 1995; Palmer and Keller, 1997). This is also the first report of the effective use of the hydrophilic polymer PEG as the sole osmoticum in microspore culture. These results should stimulate further exploration of the use of different biocompatible polymers in microspore culture systems and other similar biological systems. The basis of this approach is the utilization of polymers to meet osmotic requirements, while manipulating the sources and levels of soluble carbohydrates, which vary for specific experimental designs and microspore culture systems.

Water solubility, viscosity, inertness, biocompatibility and some other physical and chemical properties of PEG 4000 macromolecules (see Harris, 1992) make this cost effective polymer a suitable osmotic agent for microspore cultures. On the other hand, there are also disadvantages of using PEG in liquid cultures. Filter sterilization of PEG medium is a tedious

procedure, thus, filter sterilization should be avoided in protocols for scaled-up culture systems. With extended culture in a PEG medium, there is the likelihood of an accumulation of PEG within cells, and therefore prolonged culture of MD embryos on liquid PEG medium is not recommended.

Based on their study on lactate and alcohol dehydrogenases in *Phaseolus*, Boyle and Yeung (1983) suggested that the embryo environment within developing seeds was anaerobic (hypoxic) and that this anaerobic environment might play an important role in embryo development. A low oxygen supply in wheat callus cultures, a simulation of *in ovulo* conditions, promoted growth of embryonic callus and formation of somatic embryos (Carman, 1988, 1989). Oxygen availability in aqueous solutions of PEG 4000 is low (Mexal et al., 1975). Therefore, it appears that the oxygen and water restricted *in vitro* environment created by a high PEG concentration partially simulated the natural milieu within which zygotic embryos developed. However, the rather simple, hormone-free and sucrose-limited liquid medium with low O_2 and water stress created by a high concentration of PEG, still differed markedly from the complex physiological and biochemical properties of the endosperm and maternal ovular tissue. Nevertheless, it was an adequate milieu for development of MD embryos which closely resembled their zygotic counterparts, particularly from the globular to cotyledonary stage. As revealed by SEM, the external morphology of MD embryos induced on PEG and their zygotic counterparts was strikingly similar (Section 2, Fig 2.4. cf Section 4, Fig 4.2). These results challenge a traditional view that *in vitro* embryos induced from cells other than the zygote differ from zygotic embryos developed within the seed due mainly to the striking differences in their environment. Studies of embryonic mutants in *Arabidopsis* (Meinke, 1995) suggest a limited role of maternal tissue in embryo development. The majority of mutations showing altered embryo development appeared to be due to defects in genes acting within the zygote (Goldberg et al., 1994).

This detailed SEM study of MD embryo development in *B. napus* (Section 4) is the first study

in angiosperms to demonstrate a close morphological resemblance between MD embryos and zygotic embryos. The MD embryos developed on PEG were comparable in size to zygotic embryos, with normal development of two cotyledons and a smooth epidermal surface on the embryo axis. Formation of long filamentous suspensors, resembling those of zygotic embryos, was frequently observed by the end of the first week after microspore isolation. Such similarities between zygotic and non-zygotic embryos by SEM have only been reported for conifer somatic embryos (Fowke et al., 1994). In angiosperms, such as alfalfa (Xu and Bewley, 1992), substantial differences were found; somatic embryos were much larger in size than zygotic embryos throughout development and cotyledon development was delayed in somatic embryos, eventually resulting in poorly developed rudimentary cotyledons. Formation of multiple cotyledons and a very rough, malformed epidermal surface on the embryo axis in somatic embryos with formation of secondary embryos were also reported in this study. The abnormal formation of three and four cotyledons was also reported for *Theobroma cacao* somatic embryos (Dos Santos and Machado, 1989). In the earlier SEM study of pollen embryos from *Hyoscyamus niger* anther culture (Dodds and Reynolds, 1980), enlarged embryos with aberrant cotyledons and a rough epidermal surface, similar to those observed with alfalfa somatic embryos (Xu and Bewley, 1992), were illustrated.

The main differences between zygotic and MD embryos in *B. napus* were evident in the cell division patterns. A strictly determined cell division pattern existed during zygotic embryo development *in vivo*, resulting in the linear cotyledonary stage embryo with the two embryonic organs, embryo axis and cotyledons. In contrast, SEM observations indicated that such a precise cell division pattern was absent during development of MD embryos (Section 4, Fig 4.2). This observation is consistent with the histological analysis of MD embryos in *B. napus* (Yeung et al., 1996). In the present study, early embryo development from microspore and young bicellular pollen exhibited an irregular division pattern; suspensor formation was delayed and when formed, its size and shape differed, no hypophysis or its derivatives were observed, and the O′ boundary was not detectable. Nevertheless, the final cotyledon stage of

MD embryos on PEG, with body pattern consisting of all three domains, the apical, central and basal domains (West and Harada, 1993), was strikingly similar to its zygotic counterpart (Section 2, Fig 2.4.G cf. Section 4, Fig 4.2.J). It seems, therefore, that strictly determined cell divisions occurring during early embryo development, as seen in zygotic embryos developed *in vivo*, are not crucial for the establishment of the spatial organization of a developing embryo.

Interestingly, it was illustrated for the first time in somatic embryogenesis in *Arabidopsis* that early division patterns closely resemble the events during zygotic embryogenesis (Luo and Koop, 1997). In that study, however, the development of somatic embryos beyond globular structures was not reported, and therefore analysis of the entire developmental progression from a single cell to a cotyledonary stage embryo was not complete.

Pollen wall remnants were regularly found adhering to MD embryos at the root pole region and at the tips of the suspensors throughout the time course of this study. This peculiar detail was illustrated previously in studies of MD embryos in *Brassica* (for example, see Pechan et al., 1991, Fig 1; also Yeung et al., 1996, Fig 20), but was not discussed by the researchers. A link between position of these pollen wall patches and a possible origin of polarity in MD embryos was elaborated in Section 4. In the light of this SEM study, the current view which emphasizes disruption of polarity in potentially embryogenic microspores and onset of the first symmetrical division as a key event in sporophytic development (Fan et al., 1988; Zaki and Dickinson, 1991; Telmer et al., 1993, 1995) is now questioned. A more flexible model that considers a wider range of developmental stages (Binarova et al., 1997) and different pathways and fates of daughter cells in embryogenic microspores and pollen of *B. napus* is required. Based on studies of microspore embryogenesis in several species, Touraev et al. (1997) proposed such a model. The authors emphasized that stress in various forms rather than the first symmetrical division was the key factor in microspore embryogenesis. In agreement with their model, it is possible that in the experiments described here, upon heat

shock, late uninucleate microspores first divided asymmetrically forming the generative cell and the vegetative cell, which then divided repeatedly giving rise to a MD embryo. Another possibility is that the first division in embryogenic microspores, although exhibiting structural symmetry, produced two cells with different developmental fates, one giving rise to the body of the haploid embryo while the other divided to produce the susupensor. In both cases, a parallel to the situation seen after the first division of the zygote can be drawn. More information about the first division in embryogenic microspores and the fate of the vegetative and generative cells during the early events in microspore embryogenesis could be obtained using molecular and cellular markers for *Brassica*.

Suspensor formation in MD embryos has been reported previously (Pechan et al, 1991; Rahman, 1993; Yeung et al, 1996). In the present study, the formation of suspensors was observed in a large number of MD embryos. Their shape and size differed considerably: some embryos had rudimentary suspensors, while others had well developed filamentous structures, resembling the suspensors of zygotic embryos. In both cases, their formation was delayed in MD embryos, perhaps due to the constraints of the pollen wall. Suspensors usually appeared at the globular stage, after the breakdown of the pollen wall. In zygotic embryos, suspensors are derived from the basal cell (after the first asymmetrical division of the zygote) and may consist of five cells before the quadrant stage (Tykarska, 1976). They are metabolically active and have an important physiological role during early embryo development (Yeung and Meinke, 1993). In studies of *twin* mutants in *Arabidopsis* (Vernon and Meinke, 1994; Zhang and Somerville, 1997), it was proposed that the apical cell actively inhibits embryonic development of the basal cell during early embryogeny, and that an intercellular signaling mechanism between these two cells plays an important role in cell fate determination. In MD embryos, suspensors may have originated either from the generative cell in bicellular pollen or from the cell which remained quiescent after the first division in embryogenic microspore. In both cases, cell-to-cell communication between the two cells may have determined their different fates.

The increased conversion frequency of MD embryos, in both sucrose and PEG embryos, was achieved after ABA application and desiccation when compared to plantlet conversion frequency of 'immature' MD embryos (see Section 3, Fig 3.5 cf. Section 5, Fig 5.3). Since application of ABA during maturation was a requirement for induction of dessication tolerance, therefore it appears that both ABA and desiccation improved the quality and quantity of converted plantlets. Maturation drying was considered as a developmental switch that turns off embryo-specific genes and activates germination-specific genes in zygotic embryos (Finkelstein et al., 1985; Kermode, 1990). Although the moisture content of desiccated MD embryos (10-16%) was higher than the moisture content of dry seeds (7.5%), it is still considered a substantial drying which may have triggered events in MD embryos similar to those triggered by maturation drying in seeds.

Due to the minute size of PEG embryos, manipulation of individual embryos is extremely difficult and time consuming and there is a high chance of damaging the embryos by forceps or spatulas. Therefore, the use of the nylon screens of different mesh size is recommended. Also, it is important to perform each step correctly and at the proper time, particularly during the maturation, desiccation and rehydration of MD embryos. Otherwise, the conversion frequency of MD embryos could be lowered dramatically.

Manipulation of the microspore culture system as described in this study resulted in the greatly improved quality of MD embryos and consequently improved vigor of plantlets in the greenhouse. Both MD embryos and haploid plantlets of *B. napus* obtained using the low sucrose culture system with PEG as an osmoticum closely resembled zygotic embryos and seedlings. This is the first report of such a significant resemblance in angiosperm microspore embryogenesis.

6.2. Future Prospects

The results of the present study on microspore embryogenesis in *B. napus* has raised a series of questions and also revealed new directions for potential applications of the described method for microspore and embryo culture of a wider range of angiosperm species.

Sugars are important signaling molecules involved in the regulation of plant growth and development. The results of this study indicate that the presence of high level of sucrose in the medium indeed has a morphogenetic effect on MD embryos. Therefore, this microspore culture system, combined with a molecular genetics approach, appears suitable for *in vitro* studies of sugar signaling in plant embryogenesis.

Studies of events during the inductive stage of microspore embryogenesis involving a cross-disciplinary approach are required, leading toward our understanding of the molecular and cellular mechanisms which control the switch from the gametopytic to sporophytic development. The use of molecular markers in combination with cellular ones, such as the late pollen promoter-*gus* fusion gene (lat52-gus) reported in transgenic tobacco (Eady et al., 1995), would provide more information about the fate of vegetative and generative cells during the initial events in *Brassica* microspore embryogenesis.

Studies of the storage product accumulation during MD embryo maturation including patterns of protein and lipid body formation, accompanied by biochemical analysis of lipids and storage proteins, and immunolocalization of the accumulated storage proteins napin and cruciferin, would provide important information on the process of maturation in MD embryos, an area which has not yet been examined in detail.

Microspore embryos in *B. napus* provide an ideal system for studying endogenous hormone levels since this system, unlike somatic embryogenesis, does not require exogenous hormones,

except during the final maturation and desiccation stage. The hormones regulating MD embryo development are provided endogenously indicating a high autonomy of this embryogenic system. Given the difference in embryo morphology between sucrose and PEG embryos, it is reasonable to expect a difference in levels of hormones involved in the control of embryo development.

There is a considerable scope for improvements of this novel method. Such improvements include an optimization of the metabolizable carbohydrate component of the medium, including different types and levels of carbohydrates. It also includes protocol improvements, which would result in fewer manipulations *in vitro*, particularly during the maturation and desiccation phases. Microspore-derived embryos in a low sucrose culture system exhibited signs of autotrophy and this aspect needs to be examined in detail, since a mixotrophic-autotrophic microspore culture system would offer considerable advantage over the widely used heterotrophic system.

With these improvements, low sucrose culture systems involving non-permeating osmotica might be suitable for studies of *in vitro* embryogenesis with a broad range of plant species and would also have important potential applications in biotechnology and breeding programs.

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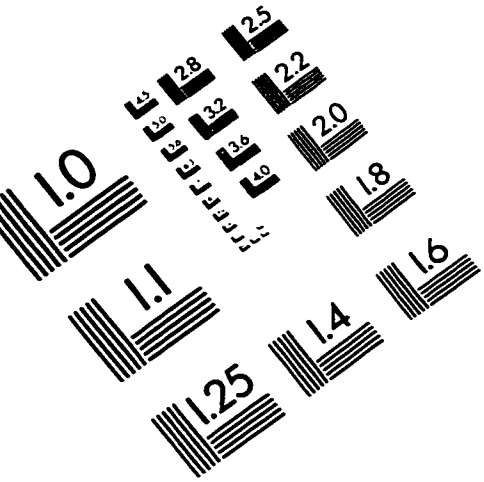
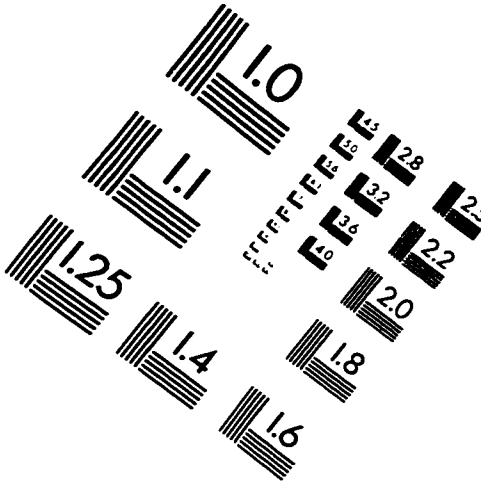
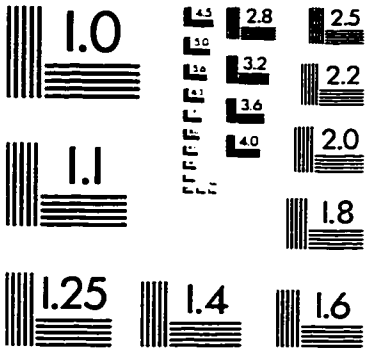
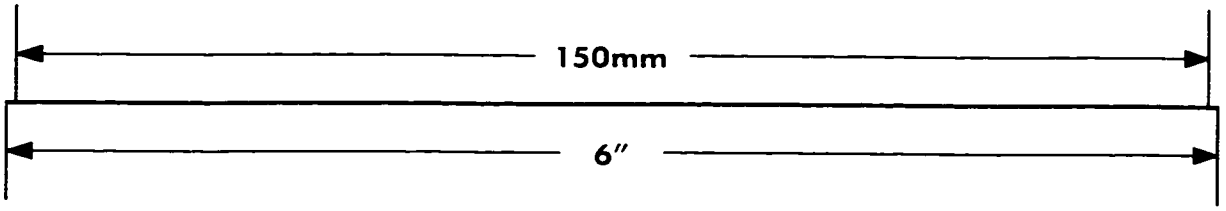
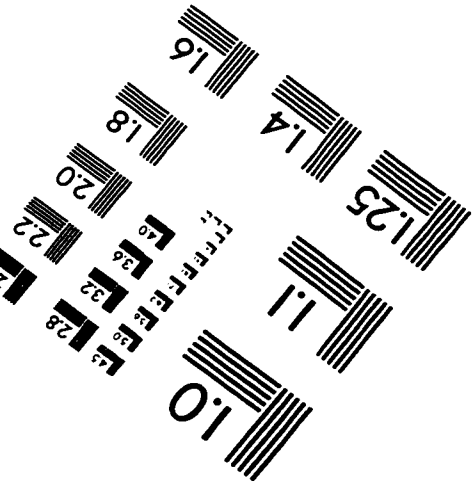
IMAGE EVALUATION TEST TARGET (QA-3)

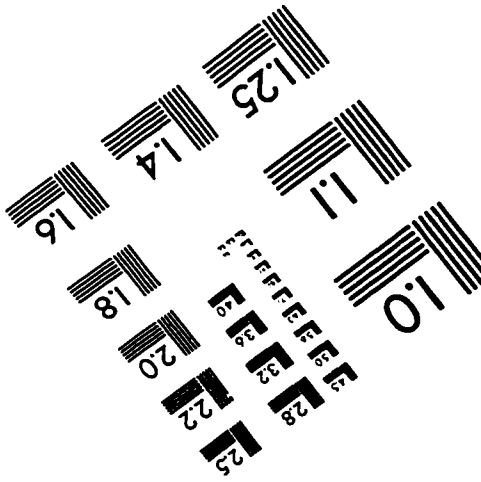












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