Innovations in microspore embryogenesis in Indonesian hot pepper (*Capsicum annuum* L.) and *Brassica napus* L.

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Innovations in microspore embryogenesis in Indonesian hot pepper (*Capsicum annuum* L.) and *Brassica napus* L.

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Bibliographic abstract

Hot pepper (*Capsicum annuum* L.) is the most important vegetable in Indonesia, but the yield is low, and the breeding programs are confined to the conventional methods and not efficient. To improve the efficiency of the breeding programs by speeding up the production of homozygous lines, studies were aimed at the introduction of haploid technology, which includes the regeneration and the production of doubled haploid plants from gametes. This technique is well developed in the model species *Brassica napus* L. via microspore culture. The results of various investigations involving both applied and fundamental aspects on microspore embryogenesis are presented in this thesis. The main results of the applied part deal with the development of an efficient shed-microspore culture protocol for the production of doubled haploid plants in Indonesian hot pepper (*C. annuum*), and its implementation under the local conditions of Indonesia. With regard to the more fundamental part, we presented for the first time an entirely new developmental pathway of embryogenesis including suspensor formation in microspore culture of *B. napus* cv. Topas that mimics zygotic embryogenesis from early stages of development onwards. These results will have significant impact for practical application in hot pepper breeding programs as well as for further fundamental research on unraveling of early plant embryogenesis.

Keywords: anther, *Brassica napus*, *Capsicum annuum*, cold-stress, doubled haploid, embryo, embryogenesis, haploid, heat-stress, hot pepper, microspore, shed-microspore, suspensor, zygotic.

Cover description:

- Front cover: An anther and embryos developed from shed-microspore culture of Indonesian hot pepper after seven weeks of culture (top) and an early globular stage embryo with suspensor from microspore culture of *Brassica napus* cv. Topas (bottom).
- Back cover: Series of photographs of a *B. napus* microspore culture showing suspensor-bearing embryo development (left), and of a shed-microspore culture in Indonesian hot pepper (right).

Contents

Preface

It dates back to 1997, when I did a small research project on anther culture of hot pepper, that I expressed my research problems to Dr. Gerard Grubben and Dr. Raoul J. Bino (CPRO-DLO, Wageningen), who at that time visited my laboratory at Bogor Agricultural University (IPB), Bogor, Indonesia. I would like to express my sincere thanks to both of them for initiating the contacts with Dr. Jan Custers, who has an extensive expertise in haploid technology. Afterwards, we prepared a research proposal 'Use of haploid technology for genetic improvement of hot pepper (*Capsicum annuum* L.)' as a part of the Biotechnology Research Indonesia-Netherlands (BIORIN) program. Meanwhile, the fellowship from the Quality for Undergraduate Education (QUE) project, Biology-IPB (IBRD LOAN No. 4193-IND) made it possible for me to come to Wageningen before the BIORIN project was started, which was September 2000. Thus, the research for this thesis was supported by the BIORIN project with the financial aid from The Royal Netherlands Academy of Arts and Sciences (KNAW), The Netherlands, and the QUE Biology-IPB fellowship Ph.D. program, Bogor, Indonesia.

This thesis was realized because of invaluable contributions of many people. Therefore I would like to express my grateful thanks.

I am very much indebted to my co-promotor, Dr. Jan Custers, for giving me the opportunity to carry out the research on 'plant haploid technology'. I learned how to carry out the basic research related to practical application, which is highly needed in Indonesia. His critical comments were very important for my learning process. His excellent supervision, continuous support and help made feasible the completion of my Ph.D. program. I also wish to express my sincere thanks to Mrs. Mieke Custers and the family members, Coen and Sanne, for their kindness and hospitality. Many thanks Jan and family!

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My grateful thanks are due to Dr. K.S. Ramulu for critically reading, discussing and correcting my manuscripts, and speeding up the realization of this thesis. Without his time and great help, the thesis would not have been the same as it looks now, or even the thesis draft should have been still lying on my desk.

I wish to express my gratitude to Dr. Bambang Suryobroto and also Dr. Aris Tjahjoleksono (Director executive QUE project, Biology-IPB) and staff, especially Yuni Maharani, for helping me to arrange everything related to my study during the preparation and for continuous help during my Ph.D. program. My grateful thanks are due to Dr. Muhamad Jusuf, Dr. Dede Setiadi, Prof. Dr. Alex Hartana (Head of my Dept. of Biology, FMIPA-IPB in succession), who gave me permission and support to study abroad. I am also thankful to Dr. Sony Suharsono and Dr. Khaswar Syamsu (Indonesian counterpart of BIORIN project from RCB-IPB) and Dr. Machmud Thohari (Director of RCB-IPB) for continuous support.

It was a very hard time to be far away from my country and family for more than four years, but the warm friendship and kindness of Indonesian students, friends and their families, especially in Wageningen, played a significant role in overcoming my home-sickness. I still could enjoy Indonesian atmosphere, the delicious home-made Indonesian food and a lot of things about Indonesian habits. Therefore, I would also like to thank pak Ruandha A. Sugardiman and family, pak Muljanto Nugroho and family, and *sadayana*. I also wish to thank the Ambassador, Consular Affairs, and Education and Cultural Affairs of Indonesian Embassy in The Hague, The Netherlands for valuable assistance and support.

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Ence Darmo Jaya Supena
Wageningen 'the city of life sciences', December 2004.

Chapter 1

General Introduction

Ence Darmo Jaya Supena

Introduction

The main aim of using an advanced technology in agriculture is to increase the productivity and quality of the crops. The first choice to achieve this aim is through the improvement of the technologies that are being used under local conditions. But, in some cases, it is highly essential to introduce and develop a new technology. In our case, the new technologies need not always to be sophisticated, but they should be more efficient and adapted for practical application under the local conditions. Haploid technology, which has a number of advantages for the improvement of crops, is one of the potential technologies in this regard. We expect that the introduction of this technology would facilitate and speed up the local crop breeding programs and cultivar development in Indonesia. In addition, improvement of haploid technology is also useful in facilitating the investigations on the basic aspects of plant genetics and embryogenesis.

Introduction, development and establishment of haploid technology for the improvement of the most important vegetable in Indonesia, hot pepper (*Capsicum annuum* L.), form the main part of this thesis. In addition, more fundamental research is carried out on new microspore embryogenesis that mimics zygotic embryogenesis in a well known model species, i.e. *Brassica napus* L. cv. Topas. The results obtained in this model system will be used for future improvement of the microspore embryogenesis in hot pepper.

Indonesian hot pepper (*Capsicum annuum* L.)

Pepper (*Capsicum annuum* L.) belongs to the genus *Capsicum* and the family Solanaceae. Pepper is closely related to the other Solanaceous crop, tomato (*Lycopersicon esculentum* Mill.). The genus *Capsicum* consists of at least 25 wild species and five domesticated ones. The domesticated species are *C. annuum* L., *C. frutescens* L., *C. chinense* Jacq., *C. bacatum* L., and *C. pubescens* Ruiz and Pav. (Anonym., 1983). Three of them, *C. annuum*, *C. frutescens* and *C. chinense* are closely related species and grouped under the *C. annuum* complex (Pickersgill, 1997). Most *Capsicum* species have 2n=2x=24 chromosomes (Anonym., 1983).

The genus *Capsicum* has originated in the American tropics, ranging from South America to Central America. The wild *C. annuum* has a wide distribution from northern Colombia to southern Arizona (Eshbaugh, 1993). Christopher Columbus brought the *Capsicum* species from Caribbean islands to Spain during his exploratory voyage in 1492-1493. Later, Portuguese traders introduced *Capsicum* in Asia; they introduced it in Indonesia in 1505. Afterwards, the pepper, especially the hot pepper types became popular in Indonesia as an important part of local cuisine spice (Berke and Shieh, 2000). Further, the *C. annuum* complex (*C. annuum*, *C. frutescens* and *C. chinense*) is grown through out the world (Pickersgill, 1997).

Djarwaningsih (1986) confirmed that the genus *Capsicum* in Indonesia was represented by five species, i.e. *C. annuum*, *C. frutescens*, *C. chinense*, *C. pubescens*, and *C. violaceum* Kunth. This classification was based on the flower and fruit characteristics of herbarium specimens preserved in Herbarium Bogoriense in Bogor, and of fresh *Capsicum* plants cultivated in Bogor Botanical Gardens and Cibodas Botanical Gardens in West Java as well as in other locations of Indonesia. According to Quagliotti (1979), the flower characteristics of *Capsicum* could be used as valid criteria for systematic classification because of wide floral characters among species and cultivars. Recently, Baral and Bosland (2002) updated the synthesis of the *Capsicum* genus, in which *C. violaceum* was identified as synonym and classified under *C. pubescens*.

Capsicum annuum and *C. frutescens* are the two species that are commonly cultivated in Indonesia, of which *C. annuum* is more important than *C. frutescens*. Two hot pepper types (*C. annuum*) that are popularly cultivated are the large type and the curly type. Both hot pepper types belong to the long cayenne fruit group (Berke and Shieh, 2000). The fruit characteristics of the large type is moderately pungent with sizes 10-13 cm long and 1.3-1.6 cm wide (e.g. 'Galaxy', 'Jatilaba' and 'Tombak' varieties), while the curly one is highly pungent with sizes 8-15 cm long and 0.5-0.7 cm wide only (e.g. 'Cemeti' and 'Laris').

Hot pepper has become the most important vegetable in Indonesia based on its economical value and cultivated area (Anonym., 2000a). However, hot pepper in Indonesia is still considered mostly as a low input crop with relatively low yield when compared to the yields of this crop in other tropical Asian countries (Anonym., 2000b). The low yield of hot pepper in Indonesia is mainly due to the heavy loss caused by pests and diseases, which also affected market quality. Anthracnose is the most serious fungal disease of hot pepper in Indonesia as well as in other tropical Asian countries, especially during the rainy season. Furthermore, the most prevalent virus diseases that infect this crop are *Chili Veinal Mottle (CVMV)*, *Cucumber Mosaic (CMV)*, *Potato Y (PVY)* and *Tobacco Mosaic (TMV)*, and the main bacterial diseases being the bacterial spot and *Phytophthora* rot. In addition, thrips, fruit flies and aphids have become the most severe insect pests (Anonym., 1988; Yoon *et al*., 1989). The resistance for pests and diseases is of polygenic nature, e.g. as found for Anthracnose (Lee and Chung, 1995; Singh *et al*., 1993) and Potyviruses (Caranta *et al*., 1997). In this regard, the local breeding programs, especially in the government research institutes, are confined to the conventional methods, which are based on direct selection procedures only. These methods are slow and inefficient for the improvement of the hot pepper crop with respect to polygenic traits and other specific adaptations needed for the local-tropical condition. Therefore, new technologies are obviously needed to speed up the breeding programs and to increase the efficiency for improving the yield and quality of hot pepper in Indonesia.

Haploid technology and its advantages

Haploid technology includes the regeneration of haploid embryos from male or female gametes and the production of haploid and doubled haploid (DH) plants from them. This technique is the most rapid route to achieve homozygosity as well as to produce pure lines. But, the spontaneous occurrence of haploids in natural populations is very rare, and it is strongly genotype-dependent. Therefore, these restricted possibilities limit the exploitation of this system. Forty years ago, Guha and Maheshwari (1964) reported a new finding. They observed numerous embryos from *in vitro* culture of anthers in *Datura innoxia* Mill. Further, they confirmed that the embryos and regenerated plants had originated from immature pollen grains with a haploid number of chromosomes (Guha and Maheshwari, 1966). This discovery has demonstrated that the male gametophytic cell has totipotency, from which the immature pollen grains could be stimulated to sporophytic divisions, which afterwards lead to the production of embryos and complete plants. Therefore, this process is designated as androgenesis. The DH plants can be produced through spontaneous doubling of haploids, or by the induction of chromosome doubling using colchicine treatment during various phases of the haploid, such as microspores, embryos, plantlets or even plants (Jansen, 1974).

In relatively a short period, the discovery of androgenesis in *D. innoxia* had a tremendous impact in stimulating further development of *in vitro* procedures for the production of haploid and doubled haploid plants in other important crops, such as tobacco (Nakata and Tanaka, 1968; Nitsch and Nitsch, 1969), barley (Kasha and Kao, 1970; Clapham, 1973), *Brassica* (Kameya and Hinata, 1970; Thomas and Wenzel, 1975), tomato (Sharp *et al*., 1972), and pepper (Wang *et al*., 1973; George and Narayanaswamy, 1973). Further, androgenesis became the most common method of choice to achieve haploidy in many crops when compared to the other available *in vitro* methods, i.e. gynogenesis and chromosome elimination (Bajaj, 1990; Ferrie *et al*., 1994, 1995; Palmer and Keller, 1999).

With the development of *in vitro* androgenesis procedures, it soon became evident that haploid and DH plants can be produced by other methods than the anther culture. For instance, androgenesis can also be induced via direct culture of isolated microspores (Reinert *et al*., 1975; Lichter, 1982) or passively by shed-microspores from anther culture in liquid medium (Ziauddin *et al*., 1990). In some species, for instance in *Brassica napus*, direct culture of isolated microspores has proven to be more efficient than anther culture for embryo production (Siebel and Pauls, 1989). The advantages of microspore culture over anther culture include: (i) a high number and more homogeneous population of microspores as starting material, (ii) the absence of growth-inhibiting substances leaking out of the degradation of anther tissue, and (iii) the absence of competition for growth, such as from the connective tissue of anther (Nitsch, 1977). Also, the other advantages are that the isolated microspore culture can be exposed directly to treatments without interfering the maternal tissue, and that the culture conditions can be controlled stringently. In some

species, for instance in *B. oleracea*, some genotypes were responsive to microspore culture in spite of their failure to respond to anther culture (Takahata and Keller, 1991; Duijs *et al*., 1992). Microspore culture was effective in avoiding the problem of albino formation in cereal anther culture (Heberle-Bors *et al*., 1996), and resulted in a high frequency of spontaneous chromosome doubling (Kasha and Maluszynski, 2003).

Doubled haploid plants have several advantages and are highly useful in facilitating the breeding programs and fundamental research in crop plants (Ferrie *et al*., 1994; Palmer and Keller, 1999). Doubled haploid plants are mostly used for parental lines in F1 hybrid variety breeding programs. Doubled haploids are also beneficial in the selection process, especially for polygenic traits, because the genetic ratio becomes simpler and fewer plants can be screened to find a particular genotype. Further, DH plants are useful in studies dealing with recessive traits, because the dominant effects do not mask the recessive-phenotype of the plant. Recently, there is also an increasing use of DH populations for molecular mapping and molecular marker-assisted selection, e.g. in pepper (Caranta *et al*., 1996, 1997, 2002; Djian-Caporalino *et al*., 2001; Lefebvre *et al*., 2002, 2003), *Brassica* (Somer *et al*., 1998; Farnham *et al*., 2002; Lionneton *et al*., 2002; Mahmood *et al*., 2003), barley (Han *et al*., 1997; Behn *et al*., 2004) and wheat (Knox *et al*., 2002; Radovanovic and Cloutier, 2003). In addition, haploid plants, which have the gametophytic number of chromosomes, serve as an important system to study mutation and selection from them (Reinert *et al*., 1975; Bajaj, 1990; Palmer and Keller, 1999).

Haploid technology has been intensively used in *Brassica* breeding and cultivar development, for instance for the breeding of *Brassica napus*, 'canola' type varieties (Hoffmann *et al*., 1982; Pauls, 1996). In *Brassica oleracea*, the breeding for resistance to *Plasmodiophora brassicae* has been facilitated enormously by using DH plants (Voorrips *et al*., 1997). From these examples, it is evident that the haploid technology will also be beneficial for hot pepper breeding in Indonesia. The technique will greatly facilitate the breeding for multiple resistances, genetic analysis of polygenic traits, rapid production of parental pure lines in F1 hybrid variety programs, and eventually it would speed up the release of new varieties adapted to local conditions.

Haploid technology in *Capsicum*

In pepper (*C. annuum*), the possibility of obtaining haploid and DH plants is known for more than sixty years. Traditionally, the haploid plants were obtained via spontaneous parthenogenesis in the form of twin embryos (Christensen and Bamford, 1943). However, the rate of haploid plants obtained through such a method was very low, approximately one per 2,000 plants, and it was strongly dependent on the genotypes, particularly restricted to sweet pepper accessions, and also dependent on the plant growth conditions (Pochard and Dumas de Vaulx, 1979). Therefore, the method of obtaining haploids spontaneously could not be exploited broadly in the pepper germplasm. Later, George and Narayanaswamy

(1973) and Wang *et al*. (1973) reported the first *in vitro* anther culture in pepper to produce haploid plants. But, the yields were still very low, and plantlets had to be regenerated via a callus phase. The more successful anther culture protocol was developed by Sibi *et al*., (1979), and was further optimised by Dumas de Vaulx *et al*. (1981). Various modified versions of this protocol have been tested, and in some cases only minor improvement was reported (Morrison *et al*., 1986; Kristiansen and Andersen, 1993; Maheswary and Mak, 1993; Qin and Rotino, 1993; Ltifi and Wenzel, 1994; Mitykó *et al*., 1995; Dolcet-Sanjuan *et al*., 1997; Gémesné *et al*., 1998; Gyulai *et al*., 2000). Further, Morrison *et al*. (1986) and later Dolcet-Sanjuan *et al*. (1997) introduced a promising double-layer medium system, liquid on the top of solid medium, for anther culture of bell pepper. On the other hand, Regner (1994, 1996) studied directly isolated microspore culture of bell pepper, but was not able to develop a successful culture protocol.

In contrast to several investigations in sweet-bell peppers, only a few studies were carried out in hot pepper genotypes. These studies indicated that hot pepper types (more spicy peppers) are less responsive in anther culture (Munyon *et al*., 1989; Qin and Rotino, 1993; Ltifi and Wenzel, 1994; Mitykó and Fári, 2001). However, further research on hot pepper genotype aimed at the improvement of protocols to efficiently exploit the ability of gametes, will certainly lead to the successful production of haploid and doubled haploid plants.

Even though the spontaneous parthenogenesis is not efficient and the existing anther culture protocols are still not efficient enough for practical application and genotype-dependent cases, they have been used to produce haploid and doubled haploid plants in some bell pepper genotypes for use in breeding programs (Dumas de Vaulx and Pochard 1986). The breeding programs were mostly for resistance to diseases and pests, such as resistance to viruses *PVY* (Pochard *et al*., 1983), *TMV* (Daubèze *et al*., 1990), *PVMP* (Caranta *et al*., 1996), *CMV* (Caranta *et al*., 2002), resistance to *Phytophthora* rot bacteria (Abak *et al*., 1982; Daubèze *et al*., 1990), bacterial spot (Hwang *et al*., 1998), powdery mildew (Lefebvre *et al*., 2003), and resistance to root-knot nematodes (Hendy *et al*., 1985; Djian-Caporalino *et al*., 2001). These suggest that the introduction of haploid technology will be beneficial to improve the efficiency of breeding programs, especially for polygenically controlled pest and disease resistances.

Brassica napus microspore culture, a model for plant embryogenesis

Haploid technology, especially developed through microspore embryogenesis, is mostly used for practical application as a tool for improving the breeding programs. However, later on, due to the inaccessibility of zygote and zygotic embryo during early development *in planta*, microspore embryogenesis has been used as a model for fundamental research on plant embryogenesis, in general. Rapeseed (*Brassica napus* L.) and tobacco are well-known model species for dicotyledonous plants, whereas barley and wheat are the models

for monocotyledonous plants. In terms of efficiency, *B. napus* microspore culture appears to be the most promising system (Swanson *et al*., 1987; Pechan and Keller, 1988). In addition, *B. napus* is closely related to the well-known plant model *Arabidopsis thaliana* (Custers *et al*., 2001).

The first isolated microspore culture protocol in *B. napus* was reported by Lichter (1982). Further, Swanson *et al*. (1987), Pechan and Keller (1988) and Custers *et al*. (1994) carried out extensive studies and developed efficient protocols of microspore culture which yielded a high frequency of haploid embryos after 10-14 days of culture. Afterwards, *B. napus* microspore embryogenesis has also been used as a model for fundamental research on molecular and biochemical analysis of early plant embryogenesis (Pechan *et al*., 1991; Boutilier *et al*., 1994, 2002; Cordewener et al., 1994, 1995, 2000). By using differential molecular screens in *B. napus* microspore culture, a number of interesting genes have been identified, which were expressed during embryo development (Boutilier *et al*., 1994; Custers *et al*., 2001). Recently, the genes *BBM* (*BABY BOOM*) and *BnCLE19* (*CLV-3/ESR*-related) involved in embryo development, were isolated and identified from *B. napus* microspore-derived embryos (Boutilier *et al*., 2002; Fiers *et al*., 2004).

From comparative morphological and histological studies in *B. napus*, Telmer *et al*. (1993), Yeung *et al*. (1996) and Ilić-Grubor *et al*. (1998) concluded that there are many similarities in morphological features of microspore-derived embryos developed *in vitro* and zygotic embryos from globular stage onwards. However, major differences were identified between microspore and zygotic embryogenesis during the early stages of embryogenesis prior to the globular stage (Figure 1). Microspore embryogenesis starts with symmetrical division (Zaki and Dickinson, 1991; Nitta *et al*., 1997), resulting in the formation of two daughter cells of equal size. These cells continue to divide to form an 'undifferentiated' cluster of cells enveloped in a microspore exine wall, which later leads to the formation of a haploid embryo sequentially (Fan *et al*., 1988; Pechan and Keller, 1988; Telmer *et al*., 1993; Hause *et al*., 1994; Yeung *et al*., 1996; Ilić-Grubor *at al*., 1998). However, a defined pattern of cell division as in the zygotic embryogenesis was not observed during early microspore embryogenesis (Custers *et al*., 2001; Yeung, 2002). Therefore, an important question addressed in this regard is whether both types of early embryogenesis are regulated by the same basic cellular mechanisms.

Besides the absence of the pattern formation, the polarity in early microspore embryogenesis is also not as clear as in zygotic embryogenesis. Nevertheless, Hause *et al*., (1994) have reported that the differential distribution of starch grains was the first sign of apical-basal polarity at the time of the rupture of the microspore exine wall. The accumulation of large starch grains was at the side of the part that eventually formed the root apex of the embryo. Further, Yeung *et al*. (1996) and Yeung (2002) indicated that the

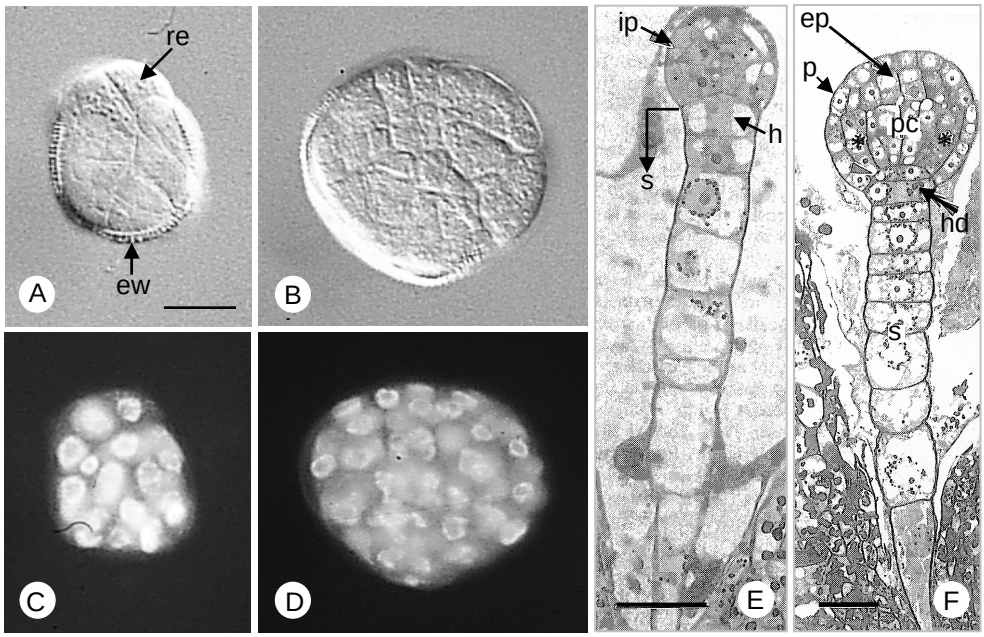

Figure 1. Early stages of embryogenesis in *Brassica napus* cv. Topas. **A-D**: Early stages of microspore embryogenesis showing the absence of pattern formation and polarity establishment. **E, F**: Early stages of zygotic embryogenesis showing well determined pattern formation and polarity establishment. **A, C**: A multicellular microspore showing the rupture at the exine-wall with at least 24 nuclei after 5 days of culture. **B, D**: An early globular embryo after 6 days of microspore culture at 32°C. **A**, **B**: Differential interference contrast micrographs (DIC) of **C**, **D** which were stained with DAPI to visualise nuclei, respectively. **E, F**: Histological sections of 5 days-old pro-embryo and early globular stages 6 days after pollination. **ew**: exine wall of microspores; **re**: rupture site of exine-wall; **s**: suspensor; **ip**: initial protoderm; **h**: hypophysis; **ep**: epiphysis; **p**: protoderm; **pc**: future procambium; *****: future ground meristem; **hd**: hypophysial cell division. Bars = 20 µm for (**A**-**D**, **E**, **F**).
(**A**-**D**: adapted from Custers *et al*., 2001 and **E**, **F**: adapted from Yeung *et al*., 1996).

existence of a suspensor-like structure can also determine the polarity in microspore-derived embryos, because this structure appeared consistently near the future root pole of the developing embryo, and the starch grains accumulated in their cells. In addition, Ilić-Grubor *et al*. (1998) have also reported that the fragments of the original microspore exine wall were consistently observed at the tips of the suspensor-like structure. Unfortunately, the appearance of a suspensor-like structure in *B. napus* microspore culture was only rarely

observed (Pechan *et al.*, 1991; Hause *et al*., 1994; Yeung *et al*. 1996; Straatman *et al*. 2000). However, Ilić-Grubor *at al.* (1998) found that the appearance of these structures varied among different experiments. They suggested that a suspensor-like structure appeared as a protuberance at the future radicle pole from the globular embryos derived from microspores. It was mostly irregular in shape when compared to the suspensor of the zygotic embryos. In fact, the suspensor in zygotic embryogenesis is one of the earliest anatomical signs of polarization of the embryo. Further investigations on early microspore embryogenesis giving rise to an embryo with suspensor-like structure will shed light on early polarity establishment and early pattern formation.

All these studies in *B. napus* indicate that the main aspect which makes early microspore embryogenesis different from zygotic situation, is the absence of the determination of pattern formation and polarity in early stages. However, the existence of a suspensor-like structure indicates the polarity establishment. A highly reproducible microspore culture, producing a high frequency of embryos with suspensor like-structure is very essential for investigations on early embryogenesis.

Aim and scope of the thesis

Haploid technology is the topic of choice for this thesis, combining both applied and more fundamental research. The applied part deals with the introduction of haploid technology for the most important vegetable, namely hot pepper (*Capsicum annuum* L.) in Indonesia, whereas the fundamental research is concentrated on microspore embryogenesis in *Brassica napus* L. cv. Topas. Thus, the research in this thesis consisted of two specific objectives:

1) To develop an efficient protocol for the production of DH plants in Indonesian hot pepper genotypes (*C. annuum*), and thereafter to implement the technology under the local conditions of Indonesia.
2) To produce the suspensor-bearing embryos reproducibly at high frequencies in a microspore culture system of the model plant species *B. napus* for a better unraveling of early embryogenesis in plants.

Chapter 2 describes various systems of anther and microspore cultures and the development of a shed-microspore culture protocol for an efficient production of DH plants in Indonesian hot pepper. Characterization and various advantages of the shed-microspore culture protocol and the response of ten genotypes of Indonesian hot pepper are presented in this chapter.

In chapter 3, several factors were investigated in order to refine the shed-microspore culture protocol in Indonesian hot pepper for improving the embryo quality in culture. The

effects of various factors are reported. In addition, the importance of using the DH lines as the donor source, is discussed.

Chapter 4 presents the data on the evaluation of several aspects to implement the shed-microspore culture and haploid technology in breeding programs of hot pepper under the local conditions of Bogor in Indonesia.

In chapter 5, the results are presented on a large-scale production of suspensor-bearing embryos from microspore culture of *B. napus*, showing a new developmental pathway that mimics zygotic embryogenesis. The initiation and function of filamentous suspensor-like structures, early pattern formation and polarity establishment in the new microspore embryogenesis are described.

Finally, in chapter 6 the importance of some factors is discussed for the development and improvement of shed microspore culture and DH plant production in Indonesian hot pepper and the perspectives of this technology in pepper breeding programs. The important finding regarding the new developmental pathway in microspore embryogenesis of *B. napus* that mimics zygotic embryogenesis, and its implications for investigations on early embryogenesis are also discussed in this chapter.

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Chapter 2

Successful development of a shed-microspore culture protocol for doubled haploid production in Indonesian hot pepper (*Capsicum annuum* L.)*

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Abstract

Various systems of anther and microspore cultures were studied to establish an efficient doubled haploid production method for Indonesian hot pepper (*Capsicum annuum* L.). A shed-microspore culture protocol was developed which outperformed all the previously reported methods of haploid production in pepper. The critical factors of the protocol are: selection of flower buds with more than 50% late unicellular microspores, a one day 4°C pretreatment of the buds, followed by culture of the anthers in double-layer medium system for one week at 9°C and thereafter at 28°C in continuous darkness. The medium contained Nitsch components and 2% maltose, with 1% activated charcoal in the solid under layer and 2.5 µM zeatin and 5 µM indole-3-acetic acid in the liquid upper layer. All the ten genotypes of hot pepper tested, responded to this protocol. The best genotypes produced four to seven plants per original flower bud. This protocol can be used as a potential tool for producing doubled haploid plants for hot pepper breeding.

Keywords: *Capsicum annuum*, hot pepper, shed-microspore culture, defective shoots

Introduction

Hot pepper (*Capsicum annuum* L.) is the most important vegetable crop in Indonesia, both in terms of cultivated area and economic value (Anonym. 2000a). However, the yield of pepper in Indonesia is relatively lower than that in other tropical Asian countries (Anonym. 2000b). Local breeding programs in Indonesia are rather traditional and generally too simple to improve the hot pepper crop with resistances against pests and diseases. There is clearly a need in Indonesia for new and more sophisticated breeding techniques for hot pepper. One such technique is haploid technology, i.e. the production of (doubled) haploid plants from male or female gametes.

Spontaneous parthenogenesis was the first system used to obtain haploid plants in bell pepper (Christensen and Bamford 1943), but the efficiency of production was low (Pochard and Dumas de Vaulx 1979). Anther culture using solid media with relatively high concentrations of growth regulators was attempted in the seventies, but yields were low and the plants had to be regenerated via a callus phase (Wang *et al*., 1973; George and Narayanaswamy 1973). Sibi *et al*. (1979) introduced a more successful two-step anther culture system, which was further optimized by Dumas de Vaulx *et al*. (1981). In this protocol, anthers are first incubated on a medium with high concentrations of growth regulators, then subcultured on a medium with low amounts of growth regulators. A heat shock (35°C) treatment was applied at the beginning of the culture. Using this protocol, the two best performing bell pepper lines gave 42 and 51 plants per 100 anthers, respectively, while a group of eight F1s yielded on average 18 plants per 100 anthers (Dumas de Vaulx *et al*., 1981). This anther culture system has been used to produce many doubled haploid plants of bell pepper for use in breeding programs (Abak *et al*., 1982; Pochard *et al*., 1983; Hendy *et al*., 1985; Dumas de Vaulx and Pochard 1986; Daubèze *et al*., 1990; Caranta *et al*., 1996).

Since the publication of Dumas de Vaulx *et al*., (1981), many other groups have attempted to use the new anther culture system for their own pepper germplasm (Vagera and Havránek 1985; Morrison *et al*., 1986; Munyon *et al*., 1989; Kristiansen and Andersen 1993; Maheswary and Mak 1993; Qin and Rotino 1993; Ltifi and Wenzel 1994; Mtykó *et al*., 1995; Dolcet-Sanjuan *et al*., 1997; Gémesné *et al*., 1998; Gyulai *et al*., 2000). But, in general, the results were disappointing, as not more than 1-5 plants per 100 anthers were obtained, and many accessions did not respond at all. Therefore, several researchers attempted to improve the method of Dumas de Vaulx *et al*. (1981). Vagera and Havránek (1985) added activated charcoal to the medium, with some beneficial effect. Morrison *et al*. (1986) subcultured the anthers after the initiation medium on a double-layer medium, i.e. solid charcoal medium with a liquid top layer. Mtykó *et al*. (1995) selected the healthy anthers every month and subcultured them on a fresh medium. Dolcet-Sanjuan *et al*. (1997) switched back from the two-step procedure to a one-step culture, using a double-layer medium with charcoal, but without growth regulators, and with maltose instead of sucrose.

These revised protocols brought about moderate improvements for the pepper genotypes investigated, but high yields as reported by Dumas de Vaulx *et al*. (1981) were not obtained. Out of more than 250 pepper accessions documented in literature, only one genotype outperformed the genotypes of Dumas de Vaulx *et al*. (1981), viz. 'Fehézörön' with 76 plants per 100 anthers (Mtykó *et al*., 1995), while another genotype 'Emerald Giant', which produced 40 plants per 100 anthers (Morrison *et al*., 1986), equalled the best genotypes of Dumas de Vaulx *et al*. (1981). In addition to the protocols described above, which are all based on anther culture systems, Regner (1994, 1996) also studied isolated microspore culture in bell pepper. He mainly used the *Brassica napus* protocol (Polsoni *et al*., 1987; Pechan and Keller 1988) together with the Dumas de Vaulx media, but was not able to develop a successful culture protocol.

In this paper, we report a procedure of shed-microspore culture for haploid plant production in Indonesian hot pepper genotypes, which are of the cayenne fruit type (Berke and Shieh 2000). We compared four different systems of anther and microspore cultures previously used in pepper and tobacco (Dumas de Vaulx *et al*., 1981; Johansson *et al*., 1982; Dolcet-Sanjuan *et al*., 1997; Touraev and Heberle-Bors, 1999) for their ability to induce embryogenesis from microspores of Indonesian hot pepper.

Materials and Methods

Plant material and microspore stage characterization

Ten Indonesian hot pepper (*Capsicum annuum* L.) genotypes were used: large hot pepper type 'Galaxy', 'Jatilaba', 'Tit-Super', 'Tombak', 'LV-2319' and 'LV-2323', and curly pepper type 'Cemeti', 'Laris', 'Tornado' and 'Typhoon'. LV-2319 and LV-2323 are breeding lines from the Indonesian Vegetable Research Institute, Bandung. The remaining genotypes are open-pollinated varieties from Indonesian seed companies. Plants were grown in glasshouses in Wageningen, The Netherlands from early April to November on 12 cm thick rockwool slabs covered with plastic. Water and nutrients were supplied by trickle irrigation, each plant having its own dripper. Temperature set points in the glasshouse were 21°C day/19°C night. A whitewash cover and an internal screen were used during summer to keep the temperature below 30°C. Plants were grown with two main stems, and young fruits were removed and plants were severely pruned to stimulate the development of new young side shoots for flower bud production. Additional light (Philips SON-T lamps) was given from early September onward to sustain plant growth.

Flower buds of the desired size (petals equal or slightly longer than sepals) were harvested in the morning, disinfected for 10 min in 2% NaOCl with 0.05% (v/v) Tween-20 added, and then rinsed three times in sterile tap water. Isolated anthers with a faint purple

tip were of the proper developmental stage, and were used for anther culture or for microspore isolation. Staining with 4', 6-diamidino-2-phenylindole (DAPI) showed that these anthers contained more than 50% of microspores in the late unicellular stage, with amounts of mid unicellular microspores and of bicellular pollen not exceeding 20% and 50%, respectively.

Culture protocols for microspore embryogenesis

Four different culture procedures were evaluated:

1. Pepper anther culture according to Dumas de Vaulx *et al*. (1981).

Anthers were incubated on solid C medium with 0.01 mg/l kinetin and 0.01 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) for 12 days, and then subcultured on R1 medium with only 0.01 mg/l kinetin. Cultures were kept at 35°C in darkness during the first eight days, and then transferred to 12 h light (25-30 $\mu mol.m^{-2}.s^{-1}$) at 25°C.

2. Pepper anther culture according to Dolcet-Sanjuan *et al*. (1997).

A double-layer medium was used, in which the under layer consisted of Nitsch components (Nitsch and Nitsch 1969) with 2% maltose and 0.5% activated charcoal (Duchefa) and was solidified with 0.6% Plant Agar (Duchefa), while the liquid top layer contained Nitsch components plus 2% maltose. Media for both layers were sterilized by autoclaving, prior to which the pH was adjusted to 5.8. Commonly, 3 cm Petri dishes were used, with approximately 1.5 ml solid medium and 1 ml liquid top layer. Cultures were kept at 9°C during the first week and then at 28°C, always in continuous darkness.

3. Tobacco anther culture according to Johansson *et al*. (1982) and Custers *et al*. (1999).

A double-layer medium system was used, in which the solid under layer consisted of half strength MS medium (Murashige and Skoog 1962) with 2% sucrose, 0.5% activated charcoal, and 0.6% Plant Agar, while the liquid upper layer contained half strength MS plus 2% sucrose. Sterilization of media and preparation of the Petri dishes were as described for the Dolcet-Sanjuan system above, with the exception that pH of the medium was adjusted to 6. Before starting the culture, flower buds were first put in a plastic jar with screw lid on a wet filter paper and stored at 9°C for seven days. The flower buds were then disinfected, the anthers isolated and incubated on top of the liquid medium. Cultures were kept at 25°C in darkness.

4. Isolated microspore culture according to the tobacco protocol by Touraev and Heberle-Bors (1999). Liquid B medium was used for the starvation of the microspores, and thereafter were subcultured into rich liquid AT3 medium. The AT3 medium contained 3, 6 or 9% sucrose or maltose as the carbon source. The pH of B medium and AT3 medium was adjusted to 6.8 and 6.2, respectively, and both liquid media were sterilised by filtration. Cultures were performed in 3 or 6 cm Petri dishes, with 1 or 3 ml microspore suspension, respectively, at a density of 40,000 microspores per ml. The microspores were cultured for six days at 25°C or 32°C, either continuously in starvation B medium or for the first 4 or 24

hours in AT3 medium containing 6% sucrose (pre-feeding) followed by culture in starvation medium. Thereafter, swollen microspores were purified by Percoll gradient centrifugation according to Kyo and Harada (1986) with 50% Percoll in 1.65x normal strength B medium and a centrifugation force of 100 g (4 min), and then transferred to rich medium and cultured at 25°C. All the cultures were maintained in continuous darkness.

Experiments performed

Especially in the Dolcet-Sanjuan procedure, we studied various conditions of certain factors in more detail.

- Flower bud cold (4°C) pretreatment was given for different periods (0, 1, 3 and 7 days) with three varieties, 'Cemeti', 'Jatilaba', and 'Tombak'. After the cold pretreatment, the anthers were isolated, and kept in culture for one week at 9°C and then at 28°C, always in continuous darkness.
- Low as well as high incubation temperatures (4°, 9°, 28°, 32°, and 35°C) were used during the first week of culture in 'Tombak'. After this treatment, all cultures were continued at 28°C, always in continuous darkness.
- Ten Indonesian cayenne pepper genotypes were compared for their performance in the system after flower bud cold (4°C) pretreatment for one day.
- The influence of different concentrations of activated charcoal from Duchefa (up to 2%) in the under layer medium was analysed in 'Tombak'.
- The effect of the addition of 2.5-25 µM zeatin or 5-50 µM indole-3-acetic acid (IAA) in liquid upper layer medium was analysed in 'Galaxy'. Also, the combinations of zeatin (2.5 or 10 µM) and IAA (5 or 25 µM) were investigated. Growth regulators were added after autoclaving.

Germination of microspore embryos

Germination medium contained half strength MS elements, 2% sucrose, and 0.1 µM 6-benzylaminopurine (BA), solidified with 0.6% Plant Agar. Cultures were performed in 6 cm Petri dishes and were kept under 16 h light (25-30 $\mu mol.m^{-2}.s^{-1}$) at 25°C. After three to four weeks, seedlings showing cotyledons and a first true leaf were transferred into honey jars with a one cm thick layer of vermiculite wet with half strength liquid MS medium supplemented with 1% sucrose. Seedlings that had formed four to six leaves were transferred to soil.

Experimental design and assessment of results

In the experiments dealing with anther culture procedures, each experiment consisted of five treatments. Per treatment, five Petri dishes were used. Each Petri dish consisted of six anthers taken randomly from six different buds. However, in the case of experiments that

were carried out to study the effect of bud pretreatment (at 4ºC) as well as the influence of genotype, anthers from one bud (mostly six anthers) were kept together in one Petri dish. Experiments were repeated at least three times during different periods. In the case of microspore culture experiments, microspores were isolated from the anthers of a batch of 20-30 buds, resulting in about 20 ml microspore suspension which was used for various treatments.

Experimental data were analysed by standard analysis of variance using General Linear Model of SPSS 10.0 for Windows software, and least significant differences (LSD) were calculated to determine the statistical significance of treatment effects. For convenience, we present the yield of embryos and plants produced per bud and not per 100 anthers, which is commonly done.

Cytological analysis

To follow early development of microspores in culture, cytological examination was conducted during the first three weeks of culture. Anthers were carefully opened with a scalpel and needles, and samples of microspores were collected for DAPI staining and observation. Upon dehiscence of the anthers, progressive development in cultures was followed by observation under a stereomicroscope.

Analysis of ploidy levels and characterization of regenerants

For ploidy analysis, a piece of leaf was cut from seedlings, nuclei were isolated and the amount of DNA was measured using a Coulter Epics XL-MCL (Beckman-Coulter, USA) flow cytometer according to the protocol described by Lanteri *et al*. (2000). Genetic proof for the gametic origin of the diploid plants derived from anther cultures was obtained by checking their selfed progeny plants for complete uniformity as compared to the heterogeneous composition of the original donor plant populations.

Results

Comparison of various culture systems

In the initial experiments, we used the Dumas de Vaulx anther culture procedure, but the results with Indonesian hot pepper were disappointing. Out of seven genotypes tested, only one was responsive and gave less than 0.2 plants per bud. The tobacco anther culture procedure of Johansson gave better results. Out of four genotypes tested, two were responsive, with an average output of 0.5 plants per bud. The best results were obtained with the Dolcet-Sanjuan system; all four genotypes tested earlier in the Johansson procedure were responsive, yielding on average 1 plant per bud.

In addition to the anther culture, we also studied isolated microspore culture (Fig. 1A). Our initial results were very promising with hot pepper. After two and a half weeks of culture, relatively high percentages (4-7%) of sporophytically dividing microspores with five to eight nuclei were found, whereas this frequency never exceeded 0.5% in the anther culture systems. In rich medium with sucrose, however, the sporophytic microspores, which contained relatively small nuclei, exhibited excessive starch accumulation and had a strong tendency to burst precociously (Fig. 1B), leading to dispersal of loose cells in the medium. This could be avoided by performing the microspore isolation in rich medium with 6% sucrose and by pre-feeding in the same medium for four hours prior to starvation in B medium. This treatment allowed sporophytic divisions to start during the starvation period. High percentages of up to 15% of sporophytic microspores were obtained, which could be increased to 40% by Percoll gradient centrifugation. Subculture in rich medium with 3 and 6% maltose stimulated the development of high quality multicellular microspores that did not show early bursting (Fig. 1C). Unfortunately, only a small percentage of the microspores successfully developed into embryos, with the maximum number of healthy plants obtained being only 0.1 per original flower bud. Despite our initial enthusiasm about isolated microspore culture, we decided to continue further experiments on the anther culture systems with a double-layer medium, especially that of Dolcet-Sanjuan.

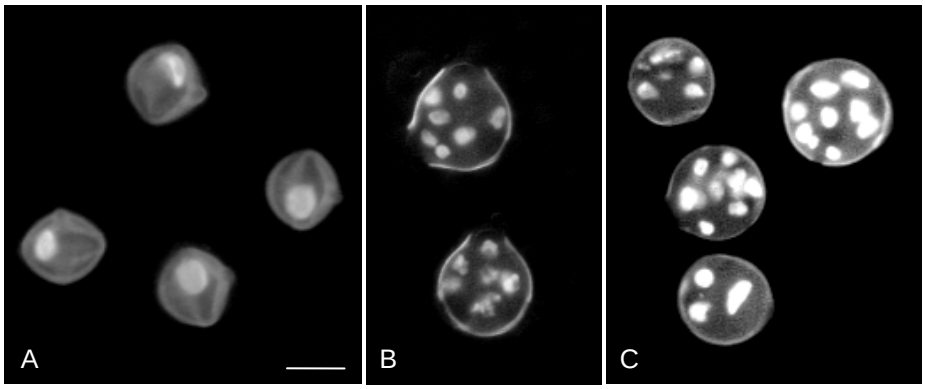

Figure 1. Early development of hot pepper (*Capsicum annuum)* 'Tombak' microspores in isolated microspore culture, as observed with DAPI staining. **A**: Freshly isolated late-unicellular microspores. **B**: Microspores with 6-8 nuclei after one week in B medium and 10 days in rich medium with 6% sucrose. The microspores show precocious bursting. **C**: Microspores with 10-14 nuclei (some nuclei out of focus) after 4 hrs pre-feeding in rich medium with 6% sucrose, six days in B medium, then 10 days in rich medium with 6% maltose. Bar = 20µm for **A**-**C**.

Anther culture resembles shed-microspore culture

Upon incubation in a double-layer culture system, the hot pepper anthers floated on the surface of the liquid upper layer medium, and after two to three weeks they showed normal dehiscence, releasing the microspores into the medium. Notably, most embryos were formed from released microspores rather than from microspores attached to the anther walls. As the development of these microspores resembles the development in shed-microspore culture, as reported for instance in barley (Ziauddin *et al*., 1990), we decided to use this term for our cultures.

Figure 2 shows early cytological observations on hot pepper 'Tombak' shed-microspore cultures. After one week of culture at 9°C, some of the microspores have started dividing sporophytically, resulting in two vegetative-like nuclei (Fig. 2A). Continuation of sporophytic divisions produced multicellular microspores with relatively large nuclei (Fig. 2B), which developed into proembryos that were first visible after three weeks of culture (Fig. 2C). The dark background of the activated charcoal layer made it difficult to detect the early proembryos with the dissection microscope (Fig. 3A), but from week 4 of culture onward, the observations became more informative. Globular to heart-shape stage embryos were visible after four weeks of culture, and had reached the torpedo to cotyledon shape after five weeks of culture (Fig. 3B). The first germination of embryos was observed one week later (Fig. 3C).

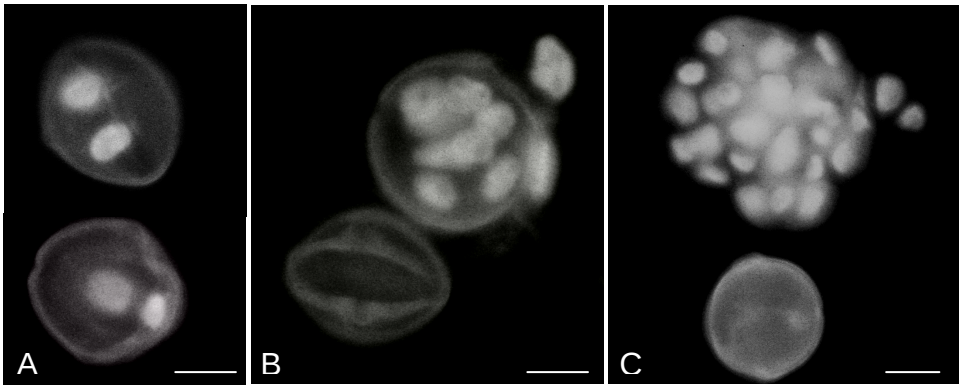

Figure 2. Early development of hot pepper (*Capsicum annuum)* 'Tombak' microspores in shed-microspore culture as observed with DAPI staining. **A** and **B**: Microspores taken from anthers incubated for one and two weeks, respectively. **C**: Proembryo after three weeks of culture that developed from a microspore dispersed in the liquid medium. Bars = 10µm for **A**-**C**.

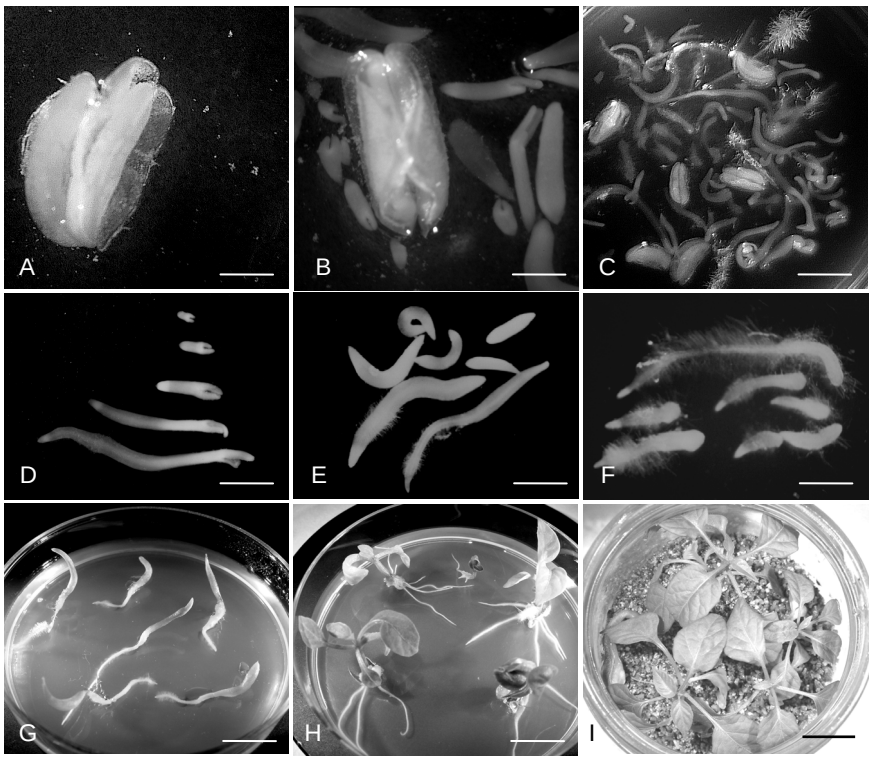

Figure 3. Time-course of events during shed-microspore culture of hot pepper (*Capsicum annuum*) 'Tombak'. **A**: Dehisced anther accompanied with proembryos in the medium after three weeks of culture. **B** and **C**: Embryos developed after five and six weeks of culture, respectively. **D-F**: Three categories of embryos obtained after seven weeks of culture: complete embryos with visible cotyledons, embryos missing cotyledons, and embryos consisting of radicles only. **G**: Pin-shaped seedlings developed from embryos without cotyledons. **H**: Normal seedlings developed from complete embryos. **I**: Plant formation on vermiculite medium. Bars = 1 mm for photographs A-B; 4 mm for C; 3 mm for D-F; 8 mm for G-H; 1.5 cm for I.

Microspore embryos differed in quality

Germination of microspore-derived embryos began after six to eight weeks of culture on embryogenesis initiation media, after which embryos were subcultured on germination medium. The germination rate was dependant on embryo quality. Actually, only approximately 20% of the embryos was normal and complete, that is consisting of radicle, hypocotyl, and cotyledons (Fig. 3D). Approximately 30% of the embryos was without cotyledons and apparently missing a shoot apical meristem (Fig. 3E), and the remaining

50% seemed to consist of a radicle only (Fig. 3F). The latter group of embryos was the first to germinate, even before subculture to germination medium, but they were incapable of producing complete seedlings. The intermediate group, lacking visible cotyledons, formed pin-shaped seedlings (Fig. 3G), of which only about 5% gave rise to normal plants, while seedling formation from the complete embryos with two cotyledons was almost hundred per cent (Fig. 3H). Well developing seedlings easily continued growth after subculture to vermiculite medium (Fig. 3I), and thereafter were successfully transferred to soil. Future experiments focused on improving the total embryo yield, as well as the yield of normal-looking embryos (complete embryos with two cotyledons and embryos with one visible cotyledon).

Temperature stress improved embryo yield

The results presented in Table 1 on the effect of temperature demonstrate that low temperatures during the first week of culture increased embryo yield, i.e. the yield of total and normal-looking embryos. The incubation for one week at 9°C gave the highest embryo yield, and therefore this temperature treatment was subsequently included as a standard treatment in our protocol. Continuous culture at 28°C gave the lowest yield of both total and normal-looking embryos. 'Tombak' donor plants in the experiment visibly varied in size, in leaf and fruit shapes, and in the amount of anthocyanin on the stems. 'Tombak' is an old landrace that is not genetically pure. The heterogeneity of the plant material was also observed in culture, giving rise to relatively high standard errors of the treatment means.

Table 1. Effect of temperature treatment during the first week of culture on embryo yield in shed-microspore culture of hot pepper (*Capsicum annuum* L.) 'Tombak'. After the temperature treatments, cultures were continued at 28°C. The experiment was repeated three times. Means within a column followed by the same letter are not significantly different at P=0.05.

Temperature (°C)	Average yield of embryos per bud	
	Total no. of embryos produced	No. of normal-looking embryos
4	13.7 ab	6.3 ab
9	22.9 a	7.8 a
28	6.1 b	3.2 b
32	12.1 b	4.4 ab
35	8.7 b	3.0 b

In addition to the 9°C treatment that gave the best results on embryo yield (see Table 1), the results obtained with 4°C were also interesting in giving high total embryo yield as well as high yield of normal-looking embryos. Therefore, we tried to exploit 4°C as cold pretreatment of the flower buds, which is a common pretreatment in tobacco anther culture. The results on the effect of different periods (0 to 7 days) of 4°C cold pretreatment on shed-microspore culture of 'Cemeti', 'Jatilaba' and 'Tombak' are shown in Figure 4. A high percentage (60-90%) of responsive buds was observed in all the three varieties. A positive effect of flower bud cold pretreatment was found for the total embryo production, but it was

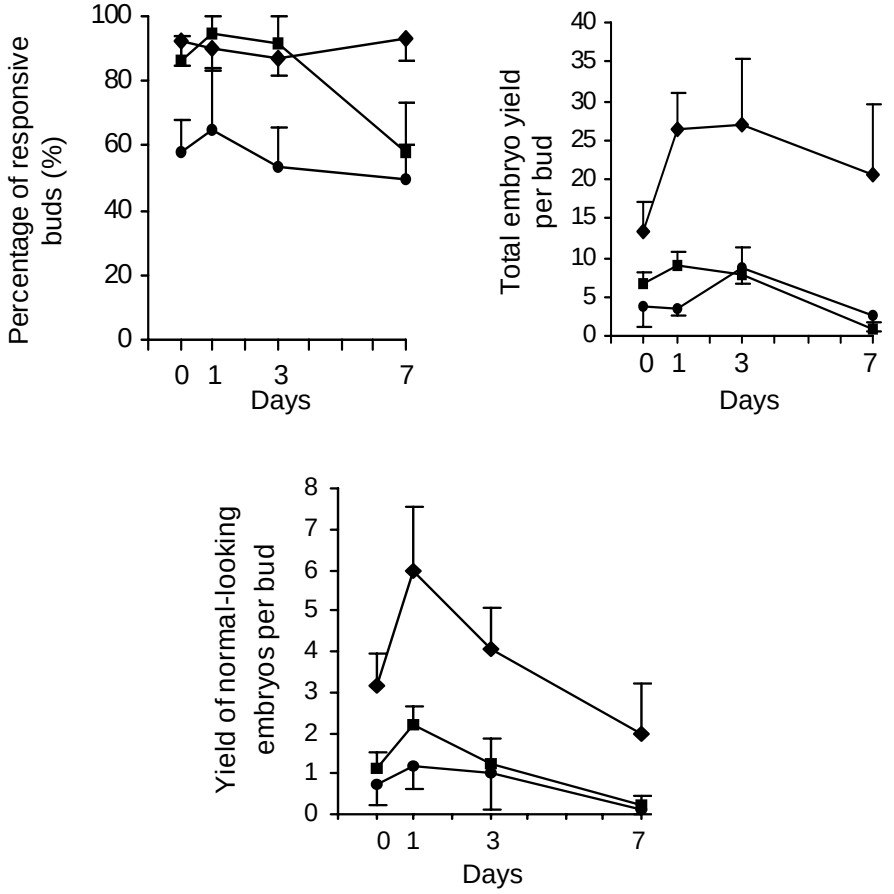

Figure 4. Effect of different periods of 4°C cold pretreatment (0, 1, 3, and 7 days) of the flower buds on shed-microspore culture of hot pepper (*Capsicum annuum)* 'Cemeti' (●), 'Jatilaba' (■), and 'Tombak' (♦). After the bud pretreatment, the anthers were kept for one week at 9°C and then at 28°C. Bud responsiveness, total embryo yield, and the yield of normal-looking embryos were analysed. Data are the means (+ or - standard errors) of three replicated experiments.

most evident for the number of normal-looking embryos. The duration of one day 4°C cold pretreatment appeared to be optimal, even though the effect was not always statistically significant, due to relatively high standard errors, as caused by heterogeneity within the donor plant populations.

In a cytological study, we examined using DAPI how one day 4°C cold pretreatment of the buds affected the early stages of microspore embryogenesis. The percentage of microspores containing various numbers of nuclei were counted during the first three weeks of culture and the data are presented in Table 2. In each treatment, the results for five buds were followed for different periods. During the first two weeks of culture, microspores were sampled from the anthers, while later after anther dehiscence, a representative part of the shed microspores was also included in the sampling. When compared to the non-pretreated culture, cold-pretreatment gave a decreased percentage of empty microspores, but increased the percentage of microspores containing more than two nuclei (sporophytically dividing microspores) after two weeks of culture. After three weeks of culture, pro-embryos (>20 nuclei) were only found in pretreated cultures (Table 2).

Table 2. Percentages of microspores containing various numbers of nuclei counted after 0, 1, 2, and 3 weeks in shed-microspore culture of hot pepper (*Capsicum annuum* L.) 'Tombak'. Shed-microspore culture was started from flower buds with or without pretreatment for one day at 4°C. Shaded areas illustrate the clearest differences between treatments. w: week.

No. of nuclei per microspore	Percentages of microspores containing various numbers of nuclei							
	in non-pretreated culture after				in 4°C pretreated culture after			
	0 w	1 w	2 w	3 w	0 w	1 w	2 w	3 w
0	0.2	2.1	42.2	75.3	0.3	1.2	23.1	77.0
1	88.7	21.6	5.7	2.7	96.3	20.9	3.4	0.2
2	11.1	75.6	43.9	17.3	3.4	77.3	58.2	16.1
3-4	0	0.7	7.9	3.8	0	0.6	14.8	5.6
5-6	0	0	0.3	0.7	0	0	0.4	0.6
7-10	0	0	0	0.1	0	0	0.1	0.2
11-20	0	0	0	0.1	0	0	0	0.3
> 20	0	0	0	0	0	0	0	0.1

Influence of genotype on shed-microspore culture response

The results of experiments on one day bud pretreatment at 4°C with ten Indonesian cayenne pepper genotypes are shown in Table 3. All the genotypes appeared responsive, but they differed in embryo yield and the production of mature plants. From the six genotypes tested for germination, two gave a high yield of more than four plants per bud, while the plant recovery rate for the other genotypes was about one plant per bud. Notably, the general performance of curly type hot pepper was lower than that of the large types. Generally, 60-75% of the normal-looking embryos gave rise to plants. The embryos with two cotyledons as well as the embryos with one cotyledon were considered as normal-looking embryos. The embryos with two cotyledons produced viable seedlings for almost 100%, while those with one cotyledon gave viable seedlings only in about 30-50% of the cases. Of the total number of embryos in different genotypes, only 15-33% yielded complete seedlings.

Table 3. Performance of six genotypes from large type and four genotypes from curly type Indonesian hot pepper (*Capsicum annuum* L.) in shed-microspore culture. Bud responsiveness, total embryo yield, yield of normal-looking embryos, and number of successfully germinated plants were analysed. The experiment was repeated three times, per genotype using each time the anthers of five buds that were pretreated for one day at 4°C.

Hot pepper genotypes (types and accessions)	Percentage of responsive buds	Average yield of embryos per incubated bud		
		Total no. of embryos produced	No. of normal-looking embryos	No. of mature plants*
Large type:				
Galaxy	93 a**	31.5 a	10.8 a	7.1
Jatilaba	91 a	8.5 bc	2.1 c	1.3
LV-2319	74 cd	11.2 bc	2.8 bc	nt
LV-2323	65 cde	4.6 c	1.7 c	nt
Tit-Super	77 bc	12.8 abc	2.2 c	nt
Tombak	90 ab	26.2 ab	5.8 b	4.3
Curly type:				
Cemeti	61 de	3.4 c	1.1 c	0.7
Laris	67 cde	3.8 c	1.3 c	1.0
Tornado	57 ef	3.3 c	1.9 bc	1.1
Typhoon	43 f	0.9 c	0.4 c	Nt

* Based on germination from at least 50 normal-looking embryos
** Means within a column followed by the same letter are not significantly different at P=0.05
nt: Not tested

Activated charcoal and growth regulators

The results on the effect of various concentrations of activated charcoal on shed-microspore culture in Indonesian hot pepper 'Tombak' are presented in Table 4. Charcoal was needed, as embryo formation almost completely failed without its addition. Both the total embryo yield and the yield of normal-looking embryos increased with increasing concentrations of charcoal from 0 to 2%. However, the effects of increasing concentrations of charcoal on both parameters did not run in parallel, and no sign of a statistical difference was observed between 1 and 2% charcoal. Two percent charcoal impaired embryonic shoot development. Consequently, activated charcoal at a concentration of 1% was chosen as standard for further experiments.

The effect of the addition of zeatin (2.5 to 25 µM) or IAA (2.5 to 50 µM) to the liquid upper layer was studied in preliminary experiments with 0.5% activated charcoal. Improved embryogenesis was found with 2.5 or 10 µM zeatin as well as with 5 or 25 µM IAA. This investigation was continued with the medium containing charcoal at 1%, focussing the attention more on the effect of different combinations of the two growth regulators. The results obtained are shown in Table 5. The combination of 2.5 µM zeatin and 5 µM IAA was very promising, as it outperformed the control treatment (i.e. without exogenous growth regulators) both in total embryo yield and in the yield of normal-looking embryos.

Table 4. Effect of activated charcoal in the solid under layer medium on embryo yield in shed-microspore culture of hot pepper (*Capsicum annuum* L.) 'Tombak'. Data are the means of three replicated experiments. Means within a column followed by the same letter are not significantly different at P=0.05.

Concentration of activated charcoal (% w/v)	Average yield of embryos per bud		
	Total no. of embryos produced	Normal-looking embryos	
		No.	%
0.00	0.2 c	0.0 c	0.0
0.25	8.7 b	1.0 c	11.5
0.50	16.6 ab	4.2 b	25.3
1.00	19.3 a	6.6 ab	34.2
2.00	22.7 a	7.1 a	31.3

Table 5. Effect of the combined addition of zeatin (Zea) and IAA in the liquid upper layer medium on embryo yield in shed-microspore culture of hot pepper (*Capsicum annuum* L.) 'Galaxy'. Data are the means of two replicated experiments. Means within a column followed by the same letter are not significantly different at P=0.05. Control: Culture without exogenous zeatin or IAA.

Treatments (μM)	Average yield of embryos per bud	
	Total no. of embryos produced	No. of normal-looking embryos
Control	25.2 b	13.0 b
Zea 2.5 + IAA 5.0	40.8 a	22.6 a
Zea 2.5 + IAA 25.0	26.0 b	17.6 ab
Zea 10.0 + IAA 5.0	28.9 b	17.8 ab
Zea 10.0 + IAA 25.0	20.9 b	12.8 b

Ploidy level of regenerated plants

Ploidy levels of plants regenerated from shed-microspore-derived embryos in six accessions were determined using flow cytometry (Table 6). The majority of the plants were haploid, indicating their origin to be from haploid microspores. The frequency of diploid plants was relatively low (14-33%) in four of the six accessions tested, whereas the other two showed 47% and 51% of spontaneous diploidization. The selfed progeny of diploid plants of 'Cemeti' and 'Tombak' were found to be highly homogeneous, whereas the original donor populations were heterogeneous. This indicated homozygosity of the diploid (doubled haploid) plants derived from shed-microspore culture.

Table 6. Ploidy analysis of plants regenerated from shed-microspore culture-derived embryos in various Indonesian hot pepper (*Capsicum annuum* L.) accessions.

Accessions	No. of plants analysed	No. of plants with various ploidy levels			
		1x	2x	3x	4x
Galaxy	29	20	9	0	0
Jatilaba	23	17	6	0	0
Tombak	39	19	20	0	0
Cemeti	32	17	15	0	0
Laris	24	15	8	1	0
Tornado	21	16	3	1	1

Discussion

In this study, we developed an efficient shed-microspore culture protocol for Indonesian cayenne type hot pepper that outperforms all the previously reported methods of haploid production in the entire pepper genus. The protocol comprises several parameters that were found to be important in earlier published *in vitro* haploid production systems with pepper (e.g. Sibi *et al*., 1979; Morrison *et al*., 1986; Vagera and Havránek 1985; Maheswary and Mak 1993; Mythili and Thomas 1995; Dolcet-Sanjuan *et al*., 1997; Gémesné *et al*., 1998), but this is the first time that these parameters have been appropriately combined together to achieve haploid production effectively. However, it is also important to consider the role of the germplasm in obtaining high embryo production and regenerants. Chillies or hot pepper, which are native to Central and South America, were introduced to South-East Asia early in the sixteenth century (Berke and Shieh 2000), and in Indonesia they likely evolved further in quite isolation from the rest of the genus. There are no reports in the literature on haploid production from Indonesian hot pepper, and since we did not test other peppers of different origins or types, it is possible that a natural genetic capacity of Indonesian hot pepper for microspore embryogenesis also contributed to the success of our protocol.

In addition to the promising shed-microspore culture system, we obtained interesting results with isolated microspore culture of hot pepper. As compared to shed-microspore culture, the change in microspore developmental fate from gametophytic to sporophytic development was much more evident in isolated microspores. Unfortunately, hardly any of the microspores developed further into embryos. Multicellular microspores in isolated microspore cultures always contained rather small nuclei, while nuclear sizes were generally large in the shed-culture multicellular microspores. Large nuclei with decondensed chromatin are usually indicative of active gene transcription, while cell groups with small nuclei are considered to be less active transcriptionally (Cooper and Hausman 2003). Therefore, we presume that the shed-culture-derived multicellular microspores with their large nuclei are more amenable to embryogenesis than the ones in the isolated microspore cultures, which likely have low or no gene activity.

We observed in the isolated microspore cultures that sporophytic divisions already started during the starvation period, if the microspores were pre-fed with medium containing 6% sucrose prior to starvation. This finding contradicts the generally accepted rule for embryogenesis induction from tobacco microspores, namely that microspores during starvation undergo a characteristic cell cycle arrest, leading to a dramatic reorganisation of cytoplasm and cytoskeleton in preparation for the first sporophytic division. Sporophytic division usually takes place in tobacco only five to seven days after subculture to rich medium (Touraev *et al*., 1996, 1997). Thus, the induction of embryogenesis by starvation stress might also occur by other ways or mechanisms, as we found for the hot pepper.

A common drawback in *in vitro* haploid procedures with pepper is the low germination success of the embryos. The problem is further increased by the transition from solid to liquid culture media, e.g. see Dumas de Vaulx *et al*. (1981) and Mitykó *et al*. (1995) versus Morrison *et al*. (1986) and Dolcet-Sanjuan *et al.* (1997). In our experiments, we also encountered this problem, which is clearly related to the occurrence of apical shoot abnormalities. Only fully normal-looking embryos with two symmetrical cotyledons were able to germinate at high frequencies into viable seedlings. Certain parameters were found to improve embryo quality in culture, namely a one day 4°C flower bud pretreatment, an increase in charcoal concentration or the addition of zeatin and IAA in the medium. However, these treatments only brought about a slight quantitative improvement and did not lead to qualitative changes in shoot formation. Recently, it became evident that defective shoot formation is also a major shortcoming in other tissue culture systems with pepper, including somatic embryogenesis (Steinitz *et al*., 2003) and organogenesis (Ochoa-Alejo and Ramírez-Malagón 2001; Wolf *et al*., 2001). For a long time, the seriousness of the problem was not apparent as earlier reports claiming successful somatic embryogenesis avoided clearly to mention the shoot abnormalities (e.g. Harini and Lakshmi-Sita 1993; Büyükalaca and Mavituma 1996; Jo *et al*., 1996). So far, no conditions have been found in pepper tissue culture research that effectively promote formation of embryos without shoot abnormalities. We have partly solved this problem, as we obtained a higher germination percentage than in earlier procedures of haploid production with pepper. Our future research will focus more on improving the embryo quality in shed-microspore culture. Obviously, the individual embryos produced in shed-microspore culture are a better experimental material to study the defective shoot problem than the clusters of somatic embryos with fused basal parts from tissue culture (Steinitz *et al*., 2003).

The results obtained in the present study clearly show that shed-microspore culture can be an attractive system for haploid production in Indonesian hot pepper genotypes. Generally, however we observed high variation within treatments, likely due to genetic differences between donor plants providing the anthers. The lack of uniformity between donor plants from a given accession was indicative of genetic heterogeneity. The aim of our study has been the establishment of a haploid production procedure for Indonesian hot pepper types, and therefore we were committed to use heterozygous local varieties, at least in the initial experiments. However, future work using homozygous doubled haploid lines produced in our laboratory as donor plants will allow us to further improve our protocol.

To conclude, in comparison with haploid production methods published earlier for pepper, we have established an efficient shed-microspore system for Indonesian hot pepper genotypes. This protocol can be used as a tool for producing doubled haploid plants and it will be implemented for routine application in breeding programs in Indonesia. We anticipate that the use of haploid technology in local breeding programs will greatly facilitate hot pepper genetic improvement in Indonesia in the near future. In addition, shed-

microspore culture can be used as a model system to study the widespread problem of defective shoot formation in pepper tissue culture.

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Chapter 3

Fine-tuning of shed-microspore culture to improve embryo quality in Indonesian hot pepper (*Capsicum annuum* L.)*

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Abstract

Low embryo quality, as indicated by a low percentage of normal-looking embryos, is a serious problem in shed-microspore culture of Indonesian hot pepper (*Capsicum annuum* L.). Therefore, several factors were investigated to refine the shed-microspore culture protocol in order to improve the embryo quality. The application of abscisic acid at 3 or 4 weeks after culture and inreased osmolality at the time of culture gave no improvement in embryo quality. The most important factors which improved the embryo quality were: a) the selection of anthers based on the appearance of a purple tip area covering 5-25% length of the anther which contained ≥60% late unicellular microspores, b) addition of 1% activated charcoal in the solid under layer medium, c) delayed enrichment with 2.5 µM zeatin and 5 µM indole-3-acetic acid in liquid upper layer medium after one or three weeks of culture, and d) the reduction of incubation temperature from 28°C to 21°C or 18°C after three weeks of culture, which resulted in a higher production (>50%) of normal-looking embryos. This improvement was possible also because of the use of doubled haploid lines as donor plants, and the distribution of anthers at random over treatments. These both factors have clearly decreased the variability within a given treatment, and improved the statistical analysis of treatment effects. The refined shed-microspore culture protocol is suitable for implementation in the breeding programs of Indonesian hot pepper.

***Keywords*:** activated charcoal, anther, embryo quality, doubled haploid, indole-3-acetic acid, zeatin.

Introduction

In vitro androgenesis through anther or microspore culture is the most rapid route and an efficient system to achieve homozygosity as well as to produce pure lines (Ferrie *et al*., 1994; Palmer and Keller, 1999). This system has been intensively used in genetic studies and breeding programs of some important crops, e.g. *Brassica napus* 'canola' type varieties (Hoffmann *et al*., 1982; Pauls, 1996). However, the drawbacks of this system for practical application in some species are mainly genotype dependency and low embryo quality with poor germination rates to produce normal plants. It is known that the low embryo quality is the most serious problem in anther culture of pepper (*Capsicum annuum* L.). A high frequency of the anther culture-derived embryos (>90%) were found to be abnormal, and failed to germinate and develop into normal plants (Sibi *et al*., 1979; Dolcet-Sanjuan *et al*., 1997). Previously, various efforts to improve the embryo quality using the most successful method of anther culture of Dumas de Vaulx *et al*. (1981) in bell pepper with some modifications of carbon source, and application of growth regulators, prolin and ascorbic acid resulted only in some minor improvements (see e.g. Gémesné *et al*., 1998; Gyulai *et al*., 2000). Also, currently it became evident that the embryo quality problem, probably due to the lack of true apical meristem development, was a major drawback in pepper somatic embryogenesis (Steinitz *et al*., 2003).

Recently, Supena *et al*. (2004) had developed an efficient shed-microspore culture protocol for the production of doubled haploid (DH) plants in Indonesian hot pepper. All the ten genotypes tested were responsive, giving rise to DH plants, and the best genotypes produced four to seven plants per original flower bud. This protocol was highly efficient when compared to the previously reported haploid production methods for pepper, but still the low embryo quality remained as the main problem. Only about 20% of the embryos were normal, and able to germinate and develop into normal plants. The remaining embryos failed to develop proper cotyledons, or they had a radicle only, and failed to produce normal plants. Further, Supena *et al*. (2004) suggested to use DH lines as donor plants for further fine-tuning of the protocol, especially to study the improvement of embryo quality.

Various approaches to improve embryo quality might include manipulations of the culture during successive steps of the microspore embryogenesis process. Evidently, the manipulation during the stress-induction step, i.e. flower bud cold pretreatment and cold incubation treatment in the first week of culture, gave beneficial effect on early stages of microspore embryogenesis in shed-microspore culture of hot pepper (Supena *et al*., 2004). The positive effects included the increased percentage of microspores with sporophytic divisions and enhanced multicellular microspore development. Manipulations during the later stages of embryogenesis, can eventually facilitate embryo development, improve embryo quality and increase the yield of embryos. The concentration of growth regulators, especially an appropriate ratio of auxin and cytokinin, plays an important role in early

embryogenesis processes. Afterwards, the application of abscisic acid and osmolality can influence embryo maturation processes both in zygotic and somatic embryogenesis (Rock and Quatrano, 1995; Stasolla and Yeung, 2003). In anther culture of potato, reduction of incubation temperature also gave beneficial effects, i.e. it increased the frequency of regeneration and shoot production (Snider and Veilleux, 1994; Rokka *et al*., 1996).

In the present study, various experiments were carried out to refine the shed-microspore culture in Indonesian hot pepper, focusing the attention mainly on the improvement of embryo quality. The results are presented on the effects of: (i) growth regulators in liquid upper layer medium in combination with an addition of activated charcoal in solid medium, (ii) the addition of abscisic acid, (iii) temporary immersion system, (iv) increased osmolality of liquid medium, and (v) reduced incubation temperature. The influences of DH plants used as the explant donor (flower buds) as well as the effect of random distribution of anthers in culture on the reduction of variability are also presented.

Materials and Methods

Plant material and microspore stage characterization

The Indonesian hot pepper (*Capsicum annuum* L) genotype 'Galaxy' and its doubled haploid (DH) plants, and 'Cemeti' were used in the present study. 'Galaxy' and 'Cemeti' are open-pollinated varieties obtained from the Indonesian seed companies, and the DH plants are the progeny of self-pollinated plants derived from shed-microspore culture. All the plants were grown in glasshouses in Wageningen, The Netherlands from early April to October according to the conditions described in Supena *et al*. (2004).

Flower buds of different sizes were used to identify various morphological characteristics related to microspore developmental stages, which were based on flower bud size, length of petals and sepals, and color of anthers. The microspore developmental stages were determined using 4', 6-diamidino-2-phenylindole (DAPI) staining.

Shed-microspore culture protocol and observations on embryo development

The shed-microspore culture protocol developed by Supena *et al*. (2004) was used to further refine the method for the improvement of embryo quality. A double-layer medium was used, in which the under layer consisted of Nitsch components (Nitsch and Nitsch 1969) with 2% maltose and 0.5 or 1% activated charcoal (Duchefa), and was solidified with 0.6% Plant Agar (Duchefa), while the liquid upper layer contained Nitsch components plus 2% maltose. The media for both layers were sterilized by autoclaving, prior to which the pH was adjusted to 5.8. Petri dishes of the size 35 x 10 mm containing approximately 1.5

ml solid under layer and 1 ml liquid upper layer medium were used. For the addition of growth regulators, they were filter sterilised and added after autoclaving.

Flower buds of the desired size (petals slightly longer than sepals) were harvested in the morning, pretreated at 4°C for one day, and later disinfected for 10 min in 2% NaOCl added with 0.05% (v/v) Tween-20, followed by rinsing 3 times in sterile tap water prior to the isolation of anthers. Afterwards, anthers were isolated according to the criterion of purple tip area and cultured at 9°C for one week and then transferred to 28°C continuously, except in the case of incubation temperature treatments, where cultures were transferred to various temperatures after two and three weeks of culture. All the incubations were carried out always in continuous darkness. After the dehiscence of the anthers in culture, two to three weeks after culture, progressive development in cultures was followed by observations under a stereomicroscope. In order to study embryo quality during the early stages, observations were carried out on embryo development under the inverted microscope by removing a small part of solid medium. Finally, the yield of normal-looking embryos and total number of embryos per bud or dish were analysed after six to eight weeks of culture.

Further, the normal-looking embryos (complete embryos with two cotyledons and embryos with one visible cotyledon) were germinated, and afterwards the seedlings were transferred to vermiculite medium and then to soil-compost medium according to the conditions described in Supena *et al*. (2004). Seeds from self pollinated DH plants were harvested and collected for further experimental purposes.

Treatments and experimental design

The effects of donor plants (original population vs. doubled haploid lines) and distribution of anthers were investigated. To improve the embryo quality, we analysed the effect of various treatments in liquid medium and diffferent culture conditions in doubled haploid lines:

a) addition of different concentrations of abscisic acid (1, 10, 50 and 100 µM) after 3 and 4 weeks of culture;
b) increased osmolality from the time of culture with mannitol (1, 2, 4, 8% (w/v)), or polyethylene glycol (PEG-3550) (2.5, 5.0, 7.5, 10% (w/v)), or Ficoll-400 (1, 2.5, 5.0, 10% (w/v));
c) using temporary immersion system in RITA® type bioreactor (Etienne and Berthouly, 2002) for observations on the later stages of embryo development;
d) a combination of activated charcoal (0.5 and 1% (w/v)) with zeatin (2.5 µM) and indole-3-acetic acid (IAA) (5 µM);
e) different periods of application of zeatin (2.5 µM) and IAA (5 µM) (0, 1, 2 and 3 weeks after culture;

f) reduced incubation temperature after two and three weeks of culture from 28ºC to 25, 23, 21, and 18ºC.

In the present study, each experiment consisted of five treatments. Per treatment, five Petri dishes (five replications) were used. Each Petri dish consisted of six anthers taken randomly from six different buds. In addition, for analysing the variability in microspore embryogenic ability among individual plants as well as between the types of distribution of anthers, anthers from one bud (mostly six anthers) were also kept together in one Petri dish. Experiments were repeated at least three times during different periods. Experimental data were analyzed by standard analysis of variance using General Linear Model of SPSS 10.0 for Windows software, and least significant differences (LSD) were calculated to determine the statistical significance of treatment effects.

Results

Doubled haploid plants are a better source for microspore embryogenesis experiments

Figure 1 gives data on the extent of variability observed in microspore embryogenic ability (i.e. yield of normal-looking embryos and total embryos) in the 'Galaxy' genotype (Gal) and the 'Galaxy-doubled haploid line-1' (Gd1) plants derived from shed-microspore culture. These results show that the variability in microspore embryogenic ability among individual plants in Gd-1 was lower than that observed in the Galaxy original population. Similar results were obtained with 'Galaxy-doubled haploid line-2' (Gd2) (data not shown). These data suggest that the genetic variability among plants within a variety could be reduced by using DH plants as the donor source. Thus, this would allow a refined analysis on the effect of treatments. There was, however, still some variation within the individual DH plants, because of the variability between developmental stages among buds.

Therefore, we carried out further experiments using DH lines Gd1 and Gd2. Figure 2 shows data on the yield of total and normal-looking embryos after the distribution of anthers from one bud randomly in different Petri dishes ('random'), or after the culture of anthers from one bud together in a Petri dish ('bud'). The data indicate that a random distribution of anthers in different dishes ('random') further reduced the variability (standard error decreased by 25% to 45%), when compared to the culture of anthers together from one bud in a dish ('bud'). On the other hand, the mean yield of normal-looking and total embryos was almost similar in both types of distributions. The mean yield of normal-looking and total embryos in both Gd1 and Gd2 were significantly higher than that in Galaxy original population.

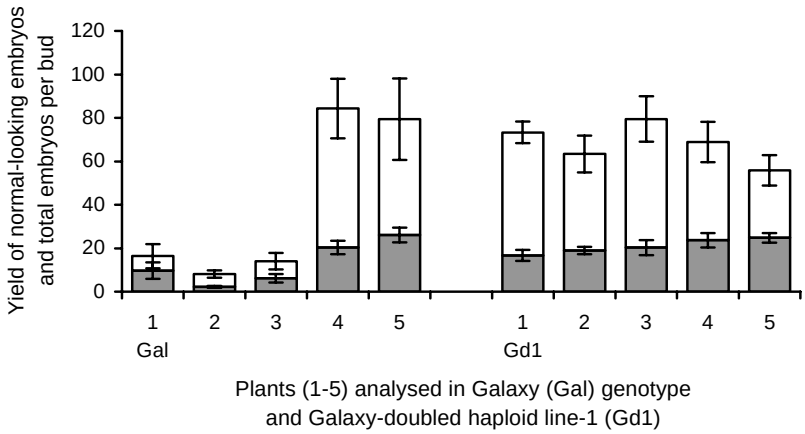

Figure 1. The variability observed in microspore embryogenic ability (i.e. yield of normal-looking embryos and total embryos) among five individual plants of 'Galaxy original population' (Gal) and 'Galaxy-doubled haploid line' (Gd1) after shed-microspore culture. The yield of total embryos (□) and normal-looking embryos (■) were analysed after 6-8 weeks of culture. The culture was with 1% activated charcoal in solid medium and without exogeneous growth regulators both in solid and liquid media. Data are the means (± standard errors) of three replicated experiments.

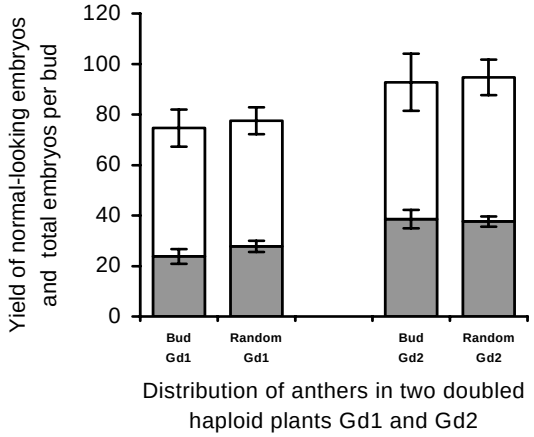

Figure 2. The yield of normal-looking and total embryos in shed-microspore culture of 'Galaxy-doubled haploid lines' (Gd1 and Gd2) after the distribution of anthers from one bud randomly in different dishes ('Random') or after the culture of anthers from one bud together in a dish ('Bud'). The yield of total embryos (□) and normal-looking embryos (■) were analysed after 6-8 weeks of culture. The culture was with 1% activated charcoal in solid medium and without exogeneous growth regulators both in solid and liquid media. Data are the means (± standard errors) of three replicated experiments.

Improvement in the selection of anthers

Using doubled haploid (DH) as the donor plants with high uniformity in morphological characteristics, we have improved the selection of starting material, which lead to high embryogenic ability. The selection is based on morphological markers of flower buds and anthers. The length of petals and sepals of buds was helpful as an early indicator for selecting the appropriate flower buds. For instance, when the length of petals is slightly longer than sepals, it could be used as an indication for the appearance of purple tips of anthers, which contained a high proportion of microspores at late unicellular stage (Fig. 3A, B). The purple color of anthers (5-25% length of the anthers) was the most effective indicator for the selection of proper starting material. With this criterion, which contained more than 60% of microspores in the late unicellular stage (Fig. 3C, Table 1), it was possible to select 100% responsive buds with embryogenic ability in Galaxy DH lines (Gd1 and Gd2) and 92% in the Galaxy genotypes (data not shown). Further, using this criterion, we have improved the responsiveness of the buds in the curly hot pepper type (Cemeti original population) up to 85%, when compared to 61% as reported earlier in Supena *et al.* (2004). On the other hand, the size of flower buds was not suitable as a morphological marker for selecting the appropriate microspore stage, because still large physiological variation occurred even in the doubled haploid lines.

Table 1. Relationship between the appearance of purple color on anthers of different bud stages and various microspore developmental stages in Galaxy-doubled haploid-1 (see Fig. 3C). EU: Early unicellular; MU: Mid-unicellular; LU: Late unicellular; EB: Early bicellular; MB: Mid-bicellular; LB: Late bicellular.

Anthers of different flower bud stages (see Fig. 3C)	Appearance of purple color on anthers	Frequencies (%) of various microspore developmental stages					
		EU	MU	LU	EB	MB	LB
1	Not yet visible	24	48	28	0	0	0
2	Just lightly initiated	0	45	55	0	0	0
3	10% length of anthers	0	5	80	15	0	0
4	25% length of anthers	0	0	60	40	0	0
5	50% length of anthers	0	0	5	50	45	0
6	100% length of anthers	0	0	0	10	35	55

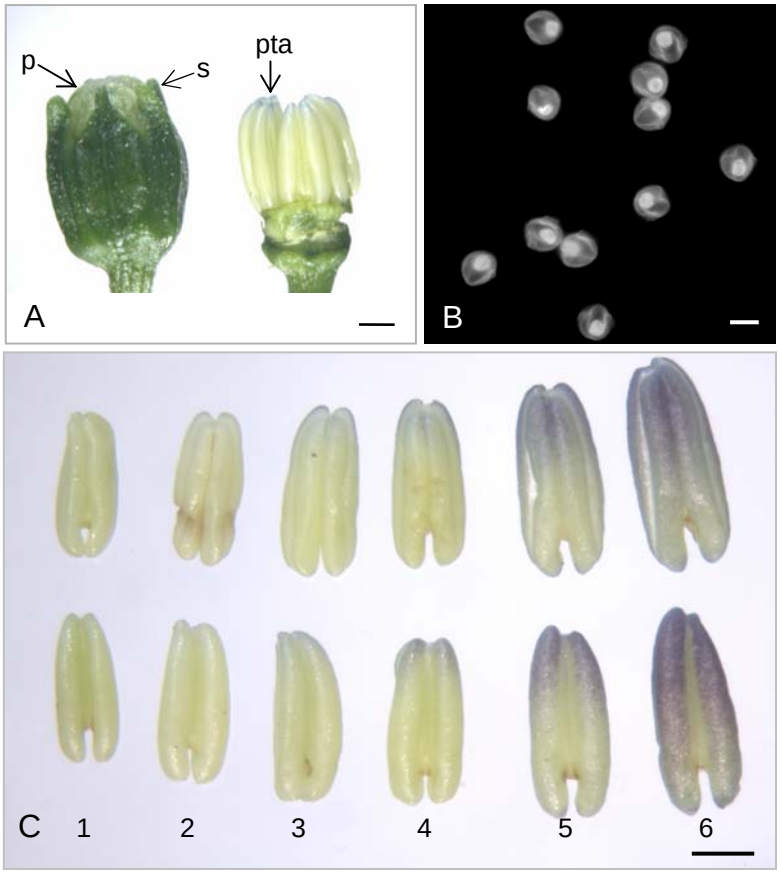

Figure 3*. Morphological markers of flower buds and anthers for various developmental stages of microspores in 'Galaxy-doubled haploid-1'. **A**: The length of petals is slightly longer than sepals and it coincides with the appearance of the purple tips of anthers. **B**: Microspores from a single bud of **A**, all showing late unicellular stage. **C**: Series of anthers from different developmental stages of flower buds showing variation of the purple color appearance; **C1**: Purple color not yet visible on the anthers; **C2**: It is just lightly initiated at the tips; **C3-6**: Anthers showing purple color on 10%, 25%, 50% and 100% of the length respectively. The relationship between the purple color of anthers and various types of microspore developmental stages of this series of anthers is presented in Table 1. Arrows point to petals (**p**), sepals (**s**), and purple tips of anthers (**pta**). Bars = 1 mm for **A** and **C**; 20 µm for **B**.

(***See appendix for color figure in page 130***)

Additive effect of activated charcoal and growth regulators on embryo yield

Table 2 gives the data on embryo yield (total number of embryos and normal-looking embryos) in shed-microspore culture of Galaxy-doubled haploid-1 after the addition of activated charcoal (AC) (0.5% and 1.0% (w/v)) alone and after the addition of AC (1.0%) together with zeatin (2.5 µM) and/or indole-3-acetic acid (IAA) (5.0 µM) at the time of culture. The results show that the increase of AC concentration from 0.5% to 1.0% significantly enhanced the total embryo yield. However, the percentage of normal-looking embryos decreased at higher concentration (1.0%) of AC. But, the enrichment of liquid upper layer medium with a combination of 2.5 µM zeatin and 5 µM IAA together with 1% AC in solid medium, significantly increased the yield of normal-looking embryos without affecting the total embryo yield. We have chosen the appropriate concentration of 2.5 µM zeatin and 5 µM IAA based on the preliminary experiments with different concentrations of zeatin (0, 1, 2.5, 5, 10 µM) and IAA (0, 2.5, 5, 10, 25 µM).

Table 2. Embryo yield in shed-microspore culture of 'Galaxy-doubled haploid-1' of Indonesian hot pepper (*Capsicum annuum* L.) after the addition of activated charcoal (AC) (0.5 and 1% (w/v)) in the solid under layer in combination with 2.5 µM zeatin (Zea) and 5.0 µM IAA in the liquid upper layer medium. Data are the means of three replicated experiments. Means within a column followed by the same letter are not significantly different at P=0.05.

Treatments	Average yield of embryos per six anthers		
	Total no. of embryos produced	Normal-looking embryos	
		No.	%
0.5% AC	56.9 b	17.3 c	30.4
1% AC	107.7 a	24.7 bc	22.9
1% AC+2.5 µM Zea	97.6 a	27.3 ab	27.9
1% AC+5.0 µM IAA	99.2 a	18.4 c	18.5
1% AC+2.5 µM Zea+5.0 µM IAA	106.4 a	35.3 a	33.2

Improvement of embryo quality

For further improvement of the quality of embryos, experiments were carried out on the effect of abscisic acid (ABA), osmolality, growth regulators and incubation temperature in shed-microspore culture of Galaxy-doubled haploid-1.

a) Effect of ABA and osmolality

The application of ABA (50 and 100 µM) to three weeks-old shed-microspore cultures resulted in the decrease of embryo quality (i.e. the yield of normal-looking embryos) and

total embryo yield, whereas it showed no effect on these when applied after four weeks of culture, except that it inhibited precocious embryo germination (data not shown). These results thus indicated that the problem of embryo quality does not occur during the maturation stages. Similarly, subculture of pro-embryo and embryo populations with the sizes >70 µm after 4-5 weeks of culture on temporary immersion system using the liquid medium with the same composition did not improve the embryo quality. Therefore, the attention was focused on early stages of embryo development.

In this regard, we carried out time course observations on embryo development under the inverted microscope after two weeks of culture. For this, a small part of solid medium containing charcoal was removed prior to the culture in order to facilitate observations in this area. These observations revealed the occurrence of embryo abnormalities during the globular stage (Fig. 4). Some embryos formed normal globular structures (Fig. 4A), while a large proportion of embryos had abnormal globular shape (Fig. 4B). This kind of abnormal globular embryos developed radicle only or embryos without cotyledon, and failed to develop into normal or complete embryos. Abnormal embryos, especially embryos with radicle only, showed precocious germination after five weeks of culture. On the other hand, about 70% of normal globular embryos developed into normal embryos with cotyledons, hypocotyl and radicle, and they did not show precocious germination. These results, thus indicate that embryo developmental problems might occur before globular stage, i.e. during the transition from multicellular microspore to early globular stages.

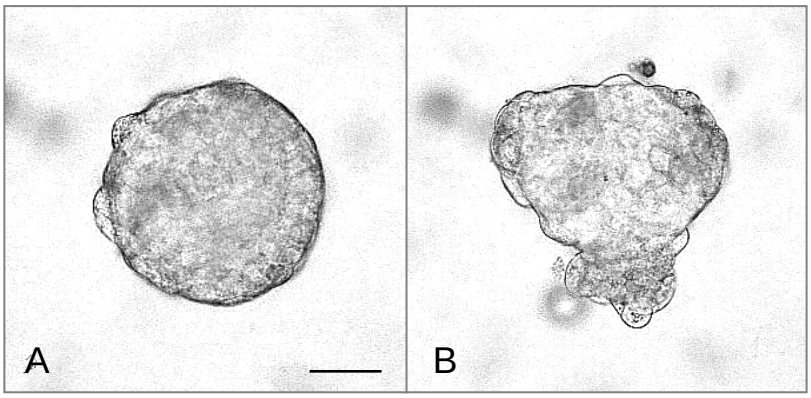

Figure 4. Normal and abnormal globular embryos in four weeks-old cultures observed under the inverted microscope in shed-microspore culture of the ‘Galaxy original population’ of Indonesian hot pepper. **A**: Normal globular embryo; 70% of this type of embryos developed into normal-looking embryos. **B**: Abnormal globular embryo; this type of embryos failed to develop into normal-looking embryos. Bar = 30 µm.

In addition to ABA treatment, we investigated the effect of the increased osmolality on microspore embryogenic ability. It was found, however, that the increase of osmolality of the medium from the time of culture using PEG-3550 or mannitol gave a detrimental effect on the yield of normal-looking embryos and total embryo yield. On the other hand, the addition of 10% (w/v) Ficoll-400 gave a slight positive effect (data not shown). This might be due to the increased viscosity of the liquid medium.

b) Effect of growth regulators

To improve embryo quality, growth regulators (zeatin and IAA) were applied at different periods of culture. Table 3 gives the data on embryo yield (total number of embryos and normal-looking embryos) after the addition of 2.5 µM zeatin in combination with 5 µM IAA at 0, 1, 2 and 3 weeks after culture. These results show that when compared to the control (i.e. without growth regulators), the addition of growth regulators at the time of culture (0 week) increased the percentage of normal-looking embryos. Further, it was observed that a delayed addition of growth regulators i.e. after 1 or 3 weeks gave still a higher percentage of normal-looking embryos. These data, thus suggest that the addition of growth regulators during an appropriate period can improve early embryogenesis development and embryo quality.

c) Effect of incubation temperature

Table 4 gives the data on the influence of the reduced incubation temperature (i.e. gradual reduction from 28°C to 18°C) after three weeks of culture. As can be seen from these results, the percentage of normal-looking embryos gradually increased. Interestingly, when the temperature was reduced to 21°C or 18°C, the frequency of normal-looking embryos increased to more than 50% (Table 4; see also Fig. 5). The total number of embryos significantly decreased with decreasing incubation temperature. On the other hand, the total number of embryos significantly decreased with the decreasing incubation temperature. In addition, the reduction of incubation temperature after two weeks of culture drastically reduced both total embryo yield and yield of normal-looking embryos (data not shown).

Table 3. Embryo yield in shed-microspore culture of 'Galaxy-doubled-haploid-1' of Indonesian hot pepper (*Capsicum annuum* L.) after the addition of zeatin 2.5 µM and IAA 5.0 µM in the liquid upper layer medium at different periods and activated charcoal 1% (w/v) in the solid under layer medium. Data are the means of three replicated experiments. Means within a column followed by the same letter are not significantly different at P=0.05.

Period (weeks after culture)	Average yield of embryos per six anthers		
	Total no. of embryos produced	Normal-looking embryos	
		No.	%
Control*	99.5	23.3 c	23.4
0	97.1	29.4 b	30.3
1	81.6	33.3 ab	40.8
2	97.0	31.0 ab	32.0
3	103.6	36.5 a	35.3

* Control was without exogenous growth regulator

Table 4. Effect of reduced temperature on embryo yield in shed-microspore culture of 'Galaxy-doubled-haploid-1' of Indonesian hot pepper (*Capsicum annuum* L.) after three weeks of culture. The culture was incubated at 9°C during the first week, 28°C during the second and third weeks of culture, and afterwards at reduced temperature of 25 to 18°C, all being in continuous darkness. Data are the means of two replicated experiments. Means within a column followed by the same letter are not significantly different at P=0.05.

Incubation temperature after three weeks of culture (°C)	Average yield of embryos per six anthers		
	Total no. of embryos produced	Normal-looking embryos	
		No.	%
28	74.7 a	20.6 a	27.5
25	52.0 b	20.2 a	38.9
23	43.6 bc	19.5 a	44.7
21	33.9 c	17.1 a	50.5
18	13.1 d	7.5 b	57.1

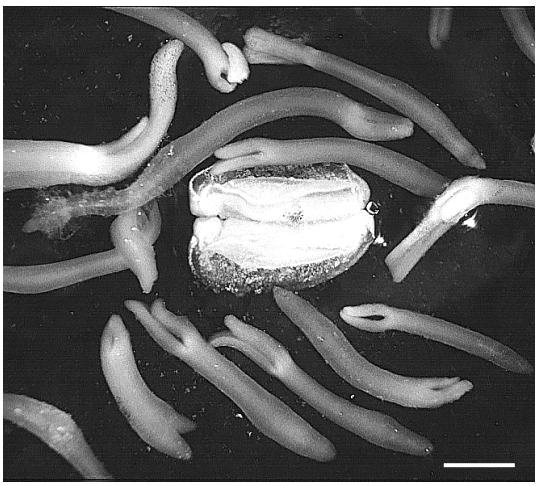

Figure 5. Normal-looking embryos observed at high percentage when the incubation temperature was reduced from 28ºC to 21ºC after three weeks of culture in shed-microspore culture of hot pepper 'Galaxy doubled-haploid-1'. The culture was seven weeks-old, with 1% activated charcoal in solid medium and without exogenous growth regulators both in solid and liquid media. Bar = 1 mm.

Discussion

The occurrence of high morphological variation among the donor plants within the genotypes in the original population of Indonesian hot pepper (*Capsicum annuum* L.) was a serious problem to obtain uniform and conclusive results on embryogenesis in shed-microspore culture (Supena *et al*., 2004). In the present study, we demonstrated that the doubled haploids can be reliably used as donor plants to solve this problem. In fact, when we used DH plants as the donor source, the yield of normal-looking and total embryos showed lower variability. This appears to be due to the high uniformity in morphological characteristics among the individual DH plants.

However, it should be mentioned here that we still observed some variation in embryo yield, which occured during different times of culture. This type of variation might be due to the physiological differences within the donor plants used. The growth conditions of donor plants, especially with regard to light and temperature, can influence the physiological status of plants.

The random distribution of anthers in different dishes for culture further reduced the variation caused by different developmental stages of flower buds. These results are consistent with Snider and Veilleux (1994), who found that the distribution of anthers from a single flower bud to different treatments had significantly reduced the variability in the anther culture of *Solanum phureja*.

In addition, an improved selection of anthers based on the level of the purple color of anthers (morphological marker), was feasible because of the high uniformity of the DH donor plants. This has further aided in performing a refined analysis by increasing the responsiveness of the flower buds as well as by narrowing the variation among different

developmental stages of flower buds used in the experiments. The purple color of anthers was the most important morphological characteristic to identify and select the proper microspore developmental stages containing more than 60% late unicellular stage. These had mid-unicellular and bicellular pollen not exceeding 20% and 40%, respectively. Similarly, in previous studies, the late unicellular to the first pollen mitotic stages have been reported as the most responsive stages for anther and microspore culture of pepper (Sibi *et al*., 1979; Gonzáles-Melendi *et al*., 1995; Barcaccia *et al*., 1999; Çiner and Tipirdamaz, 2002). However, Kim *et al*. (2004) reported that the optimal stage of anthers contains a large portion of early bicellular pollen (>75%), which differed from the findings of other authors.

Thus, the 'purple color of anthers' appeared to be the most important morphological marker for all the hot pepper accessions tested in our laboratory. Also, it is potentially suitable for other pepper types. However, in a few pepper types with white or yellow anther color (Anonym., 1995), this marker could not be used for selecting the appropriate developmental stage of the anthers. It is desirable to carry out periodically the cytological control of the microspore developmental stage using DAPI staining in order to compare with the morphological indicator.

Also the length of petals and sepals is useful in the pre-selection of flower buds in the present study. However, in some pepper types, the occurrence of variation in sepals or calyx margin (i.e. entire, intermediate, and dentate types; see Anonym., 1995) might interfere with the assessment of the length of petals and sepals.

Under the improved culture conditions, the application of increased AC concentration and addition of exogenous growth regulators (zeatin and IAA) at the time of culture significantly enhanced the embryo quality. Previously, Supena *et al*. (2004) suggested to use an increased AC concentration and the addition of zeatin and IAA as an important optional for improving the embryo quality.

Further, our attention was mainly focused on earlier stages to improve embryo quality in shed-microspore culture of hot pepper. This is due to the fact that the results on time course observations and on the effect of ABA suggested that the embryo developmental problems occur during the transition from multicellular stages to early globular stages, and not during the embryo maturation stages; our attempts to improve embryo quality after the globular stage were not successful. The application of treatments with growth regulators (zeatin and IAA) during the early stages of culture (one or three weeks of culture) showed some improvement in embryo quality.

Reduced incubation temperature after three weeks of culture considerably improved the embryo quality, i.e. the normal-looking embryos increased to >50%, but the total embryo yield decreased. It seems that the embryo developmental stages in shed-microspore culture are not uniform. A reduction of temperature might inhibit the earlier stages of embryo development, but this would be beneficial for the later stages of embryo

development. Thus, periodic isolation of individual embryos with further development from three weeks of culture, followed by subculturing at lower temperature might improve embryo quality. Previously, Snider and Veilleux (1994) and Rokka *et al*. (1996) had also reported on the beneficial effects of the reduced incubation temperature. These authors found that the reduction of incubation temperature from 28°C to 20-25°C in anther culture of potato increased the frequency of regeneration and shoot production.

In conclusion, the shed-microspore culture system refined in the present study, has significantly improved the embryo quality when compared to the protocol reported earlier (Supena *et al*., 2004). This refined system is efficient enough for the production of DH plants, and is ready for implementation to speed up the local hot pepper breeding programs in Indonesia. In addition, this system could also be potentially useful to implement in the other pepper types, such as sweet-bell pepper.

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Chapter 4

Evaluation of crucial factors for implementing shed-microspore culture of hot pepper (*Capsicum annuum* L.) in local conditions of Indonesia*

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Abstract

A shed-microspore culture protocol was developed in Wageningen for producing doubled haploid plants in several genotypes of Indonesian hot pepper (*Capsicum annuum* L.). Three crucial factors were evaluated in order to implement the shed-microspore culture of hot pepper under the local conditions of Indonesia. First, the application of a combination of the antibiotics timentin and rifampicin prevented bacterial contamination from the donor explants and eliminated the phytotoxic effects in culture, resulting in the production of embryos in three hot pepper genotypes under the local conditions of Indonesia. Second, *in vitro* application of colchicine (100 µM) during the first week of culture was highly effective in increasing the percentage of doubled haploid plants. Third, a comparative analysis of the ploidy level of plants regenerated from shed-microspore-derived embryos using chloroplast counts in guard cells of leaf stomata and flow cytometric measurement of leaf nuclear DNA content revealed that the former method is a reliable, fast and easy method in various types of Indonesian hot pepper in the local laboratories.

Keywords: *Capsicum*, contamination, chloroplast, colchicine, haploid, ploidy, rifampicin, timentin

Introduction

When *in vitro* protocols or technologies developed in sophisticated laboratories of well-developed countries are to be implemented in not fully equipped laboratories of less-developed countries, especially for practical application, then they should be adapted to local conditions. Recently, we have developed an efficient shed-microspore culture protocol for producing doubled haploid (DH) plants in various genotypes of Indonesian hot pepper (*Capsicum annuum* L.) in Wageningen, The Netherlands (Supena *et al*., 2004). This protocol is very promising to transfer to Indonesia for enhancing local breeding programs. The protocol has partly been adapted and it is easy for implementation, i.e. using autoclaved media instead of using filtered sterile media and using anthers as explants instead of isolated microspores that needs refrigerated-centrifugation in a more sophisticated haploid procedure. However, there are still some problems that can hamper the implementation of the technology under the local conditions of Indonesia, namely: (i) a high chance of microbial contamination of the cultures, (ii) non-availability of an effective in vivo method for chromosome doubling, and (iii) the absence of a reliable procedure for analysing the ploidy levels in the local laboratories.

Contamination in plant tissue culture from the donor explant source remains to be a persistent problem, especially in tropical species. This is mainly because of the difficulty to grow healthy donor plants in tropical countries. Endogenous microbial contamination is known to be the most serious problem (Kneifel and Leonhardt, 1992; Cassells, 2001). Antibiotics have been widely used to eliminate bacterial contamination problems in plant tissue culture for more than 50 years (Falkiner, 1998). But, antibiotics (e.g. rifampicin), which are effective in preventing the growth of bacteria, usually cause phytotoxic effects on plant tissue culture (Leifert *et al*., 1992; Nauerby *et al*., 1997; Teixeira da Silva *et al*., 2003). In contrast, a few antibiotics (e.g. timentin) have been reported, giving negligible negative effects, or even a minor positive effect (Nauerby *et al*., 1997; Costa *et al*., 2000). Timentin and rifampicin have a complementary effect on bactericide activity, because timentin is highly active against gram-negative bacteria (Nauerby *et al*., 1997), whereas rifampicin is more active against gram-positive bacteria (Young *et al*., 1984). In the meantime, also an interesting product Plant Preservative Mixture^{TM} (PPM) has been used for controlling the contamination problems in plant tissue culture (Fuller and Pizzey, 2001). This product was effective against bacteria and fungi (George and Tripepi, 2001; Paul *et al*., 2001). When applied in the right concentration, PPM was found to be not harmful for plant tissue culture (Compton and Koch, 2001). In the present study, we carried out experiments on the application of these antibiotics and PPM for solving the contamination problems without causing phytotoxic effect in shed-microspore culture of Indonesian hot pepper.

Another important aspect for implementing the shed-microspore culture is the availability of efficient procedure for chromosome doubling. The frequency of spontaneous

DH plants in shed-microspore culture of hot pepper was relatively low (35%) (Supena *et al*., 2004). This is similar to the results obtained in anther culture of bell pepper, i.e. 35.6% by Dumas de Vaulx *et al*. (1981), or 32.6% by Gyulai *et al*. (2000). Chromosome doubling of haploid pepper plants grown in glasshouse or in the open air is generally known to be inefficient and often troublesome (personal communication from Breeding companies). Efforts in Bogor, Indonesia to improve *in vivo* chromosome doubling in hot pepper also failed (data not published). Previous studies showed that *in vitro* colchicine treatment in pepper applied to regenerated haploid explants resulted in a 75% diploidization success rate (Mitykó and Fári, 2001). The application of colchicine during the earlier periods of culture *in vitro* had been reported as an efficient method to increase the production of DH plants in anther or microspore culture of some species, such as *Brassica napus* (Möllers *et al*., 1994; Zhao *et al*., 1996), maize (Saisingtong *et al*., 1996; Antonie-Michard and Beckert, 1997; Barnabás *et al*., 1999), and wheat (Hansen and Andersen, 1998; Redha *et al*., 1998). In the present study, the influence of *in vitro* colchicine treatment during the first weeks of culture in shed-microspore culture was investigated.

The next important step that is desirable for the implementation of *in vitro* doubled haploid production systems in practice, is to have a fast and accurate method of determining the ploidy level of microspore-derived plants. Chromosome counting from mitotic cells in root tips and from meiotic cells in flower buds is a suitable method, but difficult for tissue culture material, because plantlets have to be first grown under *in vivo* conditions, and chromosome counting also needs personnel with high-skill in cytological technique (Jansen, 1974; Wang, 1998). On the other hand, flow cytometric determination of the ploidy level is very fast, simple and accurate. However, it requires an expensive equipment. Qin and Rotino (1995) showed that the number of chloroplasts in guard cells of leaf stomata can be used to determine the ploidy levels of haploid and diploid plantlets in bell pepper. This method was also routinely used to estimate the ploidy levels of anther-culture-derived potato plants (Singsit and Veilleux, 1991) and regenerated cell- and tissue culture plants of tomato (Jacobs and Yoder, 1989; Koornneef *et al*., 1989). In the present study, we tested whether the chloroplast counting would be applicable to Indonesian hot pepper plants.

Part of the results reported in this article, especially the analyses dealing with the effect of antibiotics in preventing bacterial contamination and the application of chloroplast counts in guard cells of stomata are from the experiments carried out in Bogor, Indonesia. The other data were obtained from investigations carried out in Wageningen, The Netherlands.

Materials and Methods

Plant material and microspore stage determination

Two Indonesian hot pepper (*Capsicum annuum* L.) types were used: large types 'Galaxy' and 'Tombak' and curly types 'Cemeti' and 'Laris', all being open-pollinated varieties produced by Indonesian seed companies. Also, 'Galaxy doubled haploid-2' derived from shed-microspore culture was used. All the plants were grown in the glasshouse in Wageningen, The Netherlands from early April to October according to the conditions described in Supena *et al*. (2004). We used also other Indonesian hot pepper varieties 'Gada', 'Prabu' and 'Marathon', which were grown in the experimental station of East West Seed Company Indonesia (EWINDO), Purwakarta, West Java, Indonesia, for antibiotic experiments carried out under the local conditions of Bogor, Indonesia.

Flower buds of the desired size (petals slightly longer than sepals) were harvested in the morning, pre-treated at 4°C for one day, and then disinfected for 10 min in 2% NaOCl added with 0.05% (v/v) Tween-20, followed by rinsing for three times in sterile tap water. Anthers at proper developmental stages were chosen on the basis of the morphological indicator, i.e. faint purple tips, which contained more than 50% of microspores in the late unicellular stage, with amounts of mid unicellular microspores and of bicellular pollen not exceeding 20% and 50%, respectively (Supena *et al*., 2004).

Shed-microspore culture protocol and germination

The culture protocol developed by Supena *et al*. (2004) was used. The method is briefly outlined here. A double-layer medium was used, in which the under layer consisted of Nitsch components (Nitsch and Nitsch 1969) with 2% maltose and 0.5% activated charcoal (Duchefa), and was solidified with 0.6% Plant Agar (Duchefa). The liquid upper layer medium contained Nitsch components plus 2% maltose. Media for both layers were sterilized by autoclaving, prior to which the pH was adjusted to 5.8. Petri dishes (35 x 10 mm) were used with approximately 1.5 ml solid under layer and 1 ml liquid upper layer medium. After incubation of the anthers, cultures were kept at 9°C for one week and then transferred to 28°C continuously, always in darkness. After 7 weeks of culture, the normal-looking embryos were germinated on medium containing half strength MS elements, 2% sucrose, and 0.1 μM 6-benzylaminopurine, and solidified with 0.6% Plant Agar. Cultures were performed in 6 cm Petri dishes and were kept under 16 h light (25-30 $\mu mol.m^{-2}.s^{-1}$) at 25°C. After three to four weeks, seedlings showing cotyledons and a first true leaf were transferred into honey jars with a one cm thick layer of wet vermiculite with half strength liquid MS medium supplemented with 1% sucrose. Seedlings that had formed four to six leaves were transferred into soil-compost medium.

Antibiotic treatments, chromosome doubling and ploidy analysis

Antibiotics timentin (50 and 100 mg/l) and rifampicin (10 and 20 mg/l) alone and in combination were applied to shed-microspore culture to study their effect to prevent bacterial contamination under local conditions of Bogor Agricultural University, Bogor, Indonesia. Antibiotic treatment of rifampicin (10 mg/l) in combination with timentin (50, 100 and 200 mg/l) was also carried out at Plant Research International, Wageningen, The Netherlands to study its phytotoxic effect on embryogenic ability. In addition, we tested also the effect of a biocide Plant Preservative Mixture (0.05, 0.1, 0.2 and 0.5% v/v).

In order to develop an efficient protocol for chromosome doubling, *in vitro* application of colchicine (50 to 800 µM) during the first week of culture at 9°C was tested. After this treatment, liquid medium with colchicine was removed and immediately replaced by fresh liquid medium without colchicine and then transferred to 28°C continuously, always in darkness. Embryos were counted and classified into normal-looking embryos and total number of embryos (normal and abnormal embryos) after 7 weeks of culture. Normal-looking embryos and seedlings obtained after colchicine treatment were analysed for ploidy level. Nuclei from individual normal-looking embryos and seedlings were isolated and the amount of DNA was measured using a Coulter Epics XL-MCL (Beckman-Coulter, USA) flow cytometer at Plant Research International, Wageningen, according to the protocol described by Lanteri *et al*. (2000).

To develop an efficient method of analysing the ploidy levels, we studied the correlation between the data obtained by flow cytometric measurment of leaf nuclear DNA content and chloroplast number counts in guard cells of leaf stomata. For flow cytometric analysis of ploidy levels, leaf pieces of plantlets regenerated from shed-microspore cultures that were not treated with colchicine, were used. Afterwards, the plantlets which had been used for flow cytometric analysis were brought to Bogor, Indonesia, and eventually the mature plants (before flowering) were analysed for chloroplast numbers in guard cells of stomata. The lower epidermis of the fully expanded leaf was peeled, placed onto a microscopic glass slide, and stained with a few drops of a 1% silver-nitrate solution (Qin and Rotino, 1995). The number of chloroplasts per guard cell pair was counted under phase contrast microscope from at least ten stomata of each of three leaflet samples.

In the present study, each experiment consisted of five treatments. Per treatment, five Petri dishes were used. Each Petri dish consisted of six anthers taken randomly from six different buds. Experiments were repeated two to three times during different periods. However, for the experiments carried out in Bogor, Indonesia, a limited number of flower buds (maximum 30 buds of proper stages in each variety) were used, as supplied by the EWINDO Company. The experimental data were analysed by standard analysis of variance using General Linear Model of SPSS 10.0 for Windows software, and least significant differences (LSD) were calculated to determine the statistical significance of treatment effects.

Results

Antibiotics prevent the contamination in shed-microspore culture

The combined treatments with the antibiotics rifampicin and timentin were successfully used for preventing the bacterial contamination (73-82% contamination free-dishes) in local conditions of Bogor, Indonesia, whereas all the untreated control cultures were contaminated (Table 1 and Fig. 1). The application of timentin alone does not seem to be effective in preventing the contamination problem, whereas rifampicin alone appears to be partly effective in preventing the contamination, but gave rise to detrimental effect on microspore embryogenesis. Embryos were obtained in the contamination-free dishes in all the three genotypes tested, 'Gada', 'Prabu' and 'Marathon', especially after the combined treatment with 10 mg/l rifampicin and 50 mg/l timentin (Fig. 1E, F). Interestingly, an addition of timentin (100 mg/l) in combination with rifampicin (20 mg/l) gave some improvement on embryogenic ability compared to rifampicin alone, thus partly neutralising the negative effect of rifampicin (Fig.1, compare C with D). Further, the reduction of rifampicin (from 20 to 10 mg/1) in combination with timentin (50 mg/1) was enough to control the contamination and gave more positive effect on embryogenic ability (Fig.1 compare D with E, F).

Table 1. Effect of single treatments with rifampicin (Rif) and timentin (Tim) as well as the combined treatment of Rif and Tim for preventing the bacterial contamination in shed-microspore culture of Indonesian hot pepper (*Capsicum annuum* L.) under local conditions of Bogor, Indonesia. Data are the means of total 11 dishes per treatment of 'Gada', 'Prabu' and 'Marathon' genotypes; each dish contains six anthers from six different buds.

Antibiotic treatments (mg/l)	Contamination free-cultures (%)
Control (without antibiotics)	0
Rif 20	64
Tim 100	27
Rif 10 + Tim 50	73
Rif 20 + Tim 100	82

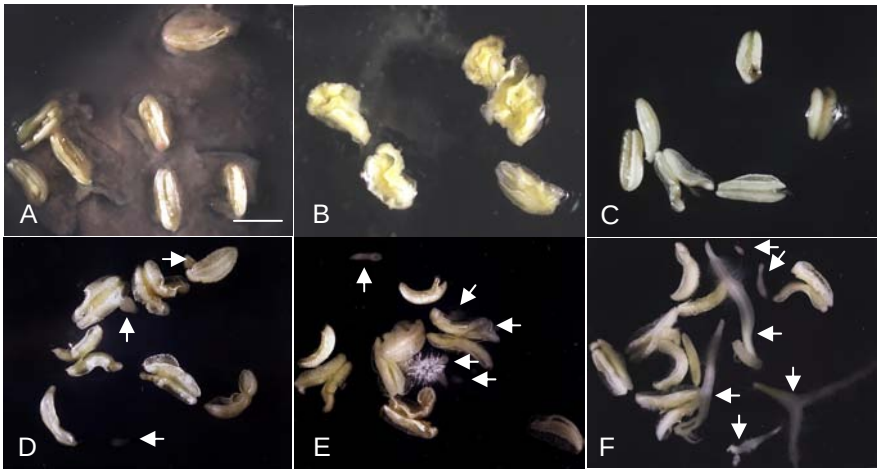

Figure 1*. Shed-microspore culture of Indonesian hot pepper (*Capsicum annuum* L.) 'Gada' after single and combination treatments of antibiotics under local conditions of Bogor, Indonesia. **A**: Control showing contaminated culture (without antibiotic treatment); **B**: Culture treated with 100 mg/l timentin showing contamination; **C**: Culture treated with 20 mg/l rifampicin showing no contamination, but without undergoing embryogenesis; **D**: Culture treated with 100 mg/l timentin + 20 mg/l rifampicin showing no contamination, where some embryos can be seen (arrows); **E-F**: Culture treated with 50 mg/l timentin + 10 mg/l rifampicin showing no contamination, where more embryos can be seen (arrows, some embryos out of focus). The culture was with 0.5% activated charcoal in solid medium and without exogeneous growth regulators both in solid and liquid media; A-E: Five weeks after culture; F: Six weeks after culture. Bar = 4 mm for A-F.
(***See appendix for color figure in page 130***)

To enhance the positive effect of timentin (i.e. for reducing the negative effect of rifampicin) on embryo yield, we studied the effect of various concentrations of timentin (50, 100 and 200 mg/l) in combination with rifampicin (10 mg/l) in shed microspore culture of 'Tombak' in Wageningen (Fig. 2). The results confirmed the highly beneficial effect of the combination treatment with 200 mg/l timentin and 10 mg/l rifampicin, where timentin almost completely neutralised the phytotoxic effect of rifampicin on embryogenic ability, i.e. the yield of total and normal-looking embryos.

In addition, we tested the effect of 'Plant Preservative Mixture' (PPM), which was principally designed to inhibit airborne contamination from both bacteria and fungi (Plant Cell Technology, Inc.). The results presented in Figure 3 show that the addition of PPM in the medium at concentrations of 0.05-0.1% (v/v) did not significantly affect the yield of normal-looking embryos and total embryos. On the other hand, the treatment at 0.2% or 0.5% resulted in phytotoxic effects, reducing the embryo yield. The particular concentration range of PPM (i.e. 0.05-0.1%) is not toxic for microspore embryogenesis, and this range is

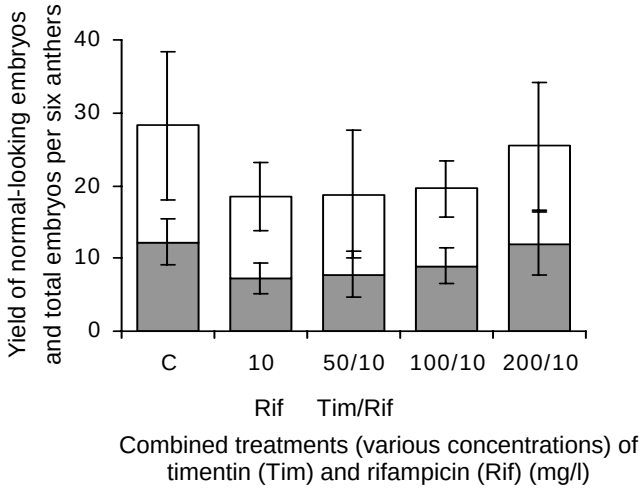

Figure 2. The effect of various timentin concentrations (50, 100 and 200 mg/l) in combination with 10 mg/l rifampicin on shed-microspore culture of hot pepper (*Capsicum annuum* L.) 'Tombak'. The yield of total embryos (□) and normal-looking embryos (■) was determined after 7 weeks of culture. The culture was with 1% activated charcoal in solid medium and without exogeneous growth regulators both in solid and liquid media. Data are the means (± standard errors) of three replicated experiments. C: Control; Tim/Rif: Combined treatment of timentin and rifampicin.

recommended to prevent airborne contamination by the manufacturer (http://www.ppm4plant-tc.com/instructions.htm). Therefore, it is potentially useful for further tests under local conditions of Indonesia, especially to prevent fungal contamination.

In vitro application of colchicine treatment increases DH plant production

A protocol for producing DH plants *in vitro* is considered as efficient when it increases not only the frequency of plants produced, but also the frequency of spontaneous diploidization in the system. In order to develop such a protocol for chromosome doubling, *in vitro* application of colchicine treatments at various concentrations, i.e. 50 to 800 µM was tested in 'Galaxy' (Fig. 4). We found that the treatment with 100 µM colchicine during the first week of culture *in vitro* was effective in increasing DH plant production. The percentage of DH plants increased by almost two-fold (i.e. from 33% to 61%) without severely decreasing the embryo yield, especially the production of normal-looking embryos. The application of colchicine at a lower concentration (50 µM) did not significantly increase the percentage of DH plants, whereas the colchicine treatment applied at higher concentration (≥200 µM) caused detrimental effect on the yield of normal-looking embryos and total

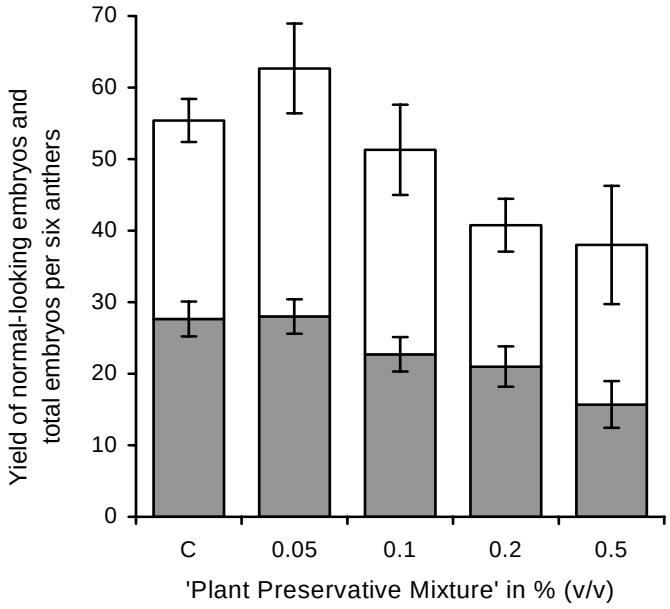

Figure 3. The effect of 'Plant Preservative Mixture' (0.05% to 0.5%) on shed-microspore culture of hot pepper (*Capsicum annuum* L.) 'Galaxy doubled haploid-2'. The yield of total embryos (□) and the normal-looking embryos (■) was determined after 6-8 weeks of culture. The culture was with 1% activated charcoal in solid medium and without exogeneous growth regulators both in solid and liquid media. Data are the means (± standard errors) of three replicated experiments. C: Control.

embryos. It should be mentioned here that this is the first time to report on *in vitro* application of colchicine in microspore embryogenesis of pepper, and that too with high success on the production of DH plants. Observations of some diploid plants derived from shed-microspore culture treated with colchicine (100µM) revealed normal fertility, which is similar to that observed in DH plants regenerated from shed-microspore culture not treated with colchicine (data not shown).

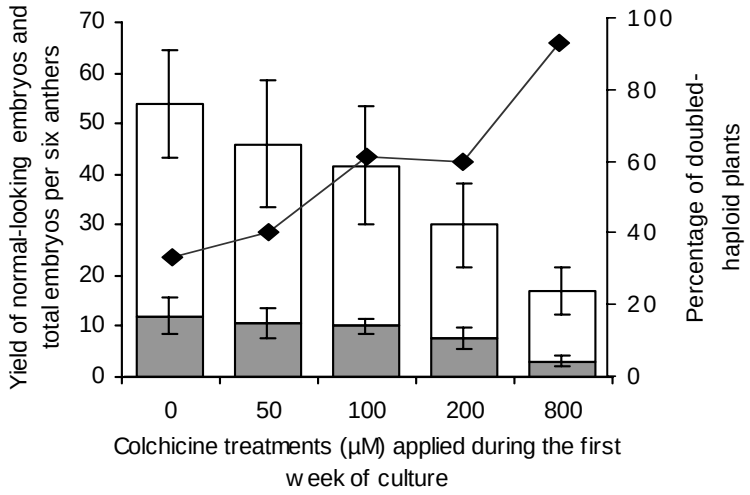

Figure 4. The effect of colchicine treatment applied during the first week of shed-microspore culture of hot pepper (*Capsicum annuum* L.) 'Galaxy'. The yield of total embryos (□) and normal-looking embryos (■) was determined after 6-8 weeks of culture. The culture was with 0.5% activated charcoal in solid medium and without exogeneous growth regulators both in solid and liquid media. Data are the means (± standard errors) of three replicated experiments. The ploidy levels of at least 15 seedlings derived from normal-looking embryos in each treatment were analysed by flow cytometry and the percentage of DH plants (—♦—♦—) was calculated.

Rapid analysis of ploidy levels of plants

In order to have a more practical method of analysing the ploidy levels, we have studied two methods, i.e. 1) flow cytometric measurement of leaf nuclear DNA content and 2) chloroplast counts in guard cells of leaf stomata. The plantlets obtained from shed-microspore culture were analysed by flow cytometry, and eventually the mature plants (before flowering) were analysed for chloroplast numbers. The comparison of results presented in Table 2 and Figure 5 clearly show a positive relationship between the chloroplast number in guard cells of stomata and the ploidy levels determined by flow cytometry. The chloroplast number of DH plants had almost double the number of chloroplasts in haploid plants. Thus, the haploid plants can be easily distinguished from the DH plants obtained from shed-microspore culture-derived embryos. Also, the number of chloroplasts per guard cell pair in DH plants and the diploid plants grown from seeds was similar. The chloroplast number was also similar ($p=0.05$) among varieties with the same ploidy level (i.e. large type and curly type). In mature, vegetatively grown plants, haploids showed smaller and narrower leaves than those of DH plants regenerated from shed-microspore-derived embryos of the same genotype (Fig. 5G).

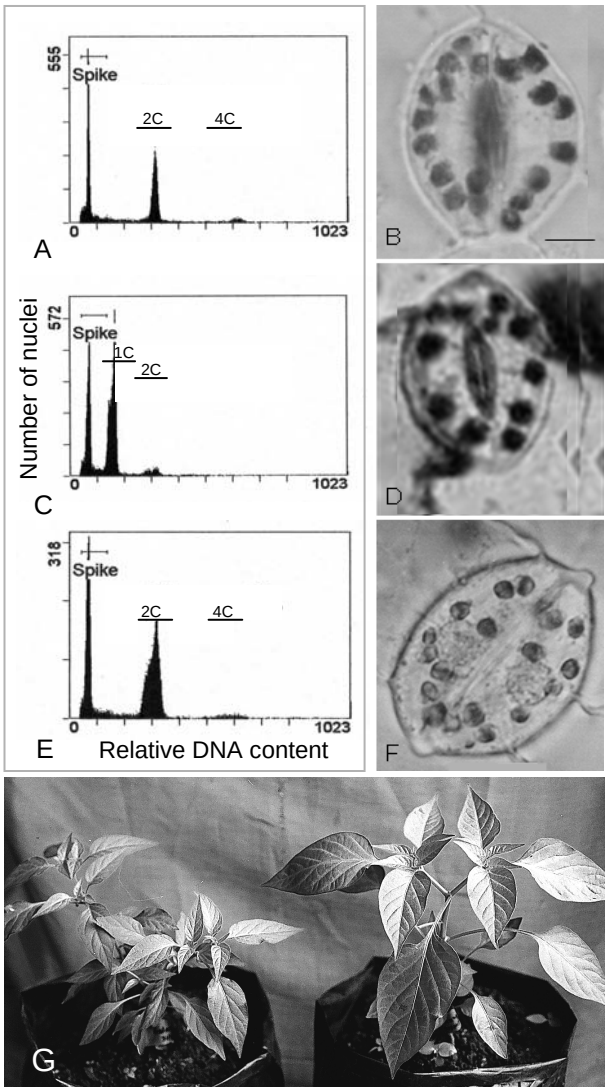

Figure 5. Analysis of ploidy level of plants by flow cytometric determination of leaf nuclear DNA contents (**A**, **C**, **E**) and by chloroplast counts in guard cells of leaf stomata (**B**, **D**, **F**). **A**-**B**: Diploid plant derived from seeds of 'Cemeti'; **C**-**D**: Microspore-derived haploid plant of 'Cemeti'; **E**-**F**: Microspore-derived DH plant of 'Cemeti'; **G**: Phenotype of microspore-derived haploid (left) and DH (right) plants of 'Galaxy'. 1C and 2C DNA contents refer to the G1 and G2 phases in microspore-derived haploid plants; 2C and 4C DNA contents refer to the G1 and G2 phases in diploid plants obtained from seeds and DH plants from microspore-derived embryos. Spike refers to the DNA content in seeds (G1 phase) of *Brassica napus*, which was used as a standard (about 2.3 pg/nucleus). The DNA content of 2C (G1 phase) in diploid plants of 'Cemeti' obtained from seeds is about 8.1 pg/nucleus. Bar = 7 µm for **B**, **D**, and **F**.

Table 2. Chloroplast number in guard cells of stomata in four varieties of Indonesian hot pepper (*Capsicum annuum* L.). Haploid and doubled haploid plants were regenerated from shed-microspore-culture-derived embryos and the ploidy levels were determined by flow cytometry analysis, whereas the diploid plants were grown from seeds.

Hot pepper varieties	Chloroplast number per guard cell pair*		
	Haploids	Doubled haploids	Diploids
Large type:			
Galaxy	9.0 a**	16.3 b	16.7 b
Tombak	8.9 a	15.8 b	16.5 b
Curly type:			
Cemeti	9.2 a	17.1 b	17.3 b
Laris	9.6 a	17.4 b	17.3 b

* Chloroplast number per guard cell pair was based on the analysis of 30 stomata from three leaflets.

* * Mean of chloroplast numbers followed by the same letters was not significantly different at p = 0.05.

Discussion

The results obtained in the present study show that the combination treatment of timentin and rifampicin was highly effective in preventing the bacterial contamination in local conditions of Bogor, Indonesia. After applying this treatment, embryos were produced from contamination-free cultures. One possible reason for overcoming the bacterial contamination problem in shed-microspore culture is that timentin and rifampicin have a complementary effect on bactericide activity against both gram-negative and gram-positive bacteria (Nauerby *et al*., 1997; Young *et al*., 1984). Further, this antibiotic combination gave a highly beneficial effect in reducing the phytotoxic effect on embryogenic ability, i.e. on the yield of normal and total embryos. In addition, the application of 0.05-1.0% PPM treatment in liquid medium seemed to be an effective alternative to prevent the contamination from airborne sources, bacterial and especially fungal contaminations.

Further, the present data revealed that when the microspore cultures were not treated with antibiotics under the local conditions of Bogor, they were all contaminated. This indicates that growing healthy donor plants in tropical environment is very difficult, and moreover the endogenous microbial contamination is a serious problem. Although the combination treatment timentin and rifampicin was highly effective in preventing the contamination problem, it should be noted here that the application of antibiotics in tissue culture has only a bacteriostatic function. Therefore, the repeated use of antibiotics in plant

tissue culture may cause resistance development by the bacteria (Kneifel and Leonhardt, 1992). The best way in plant tissue culture is to use healthy donor plant material. Fully controlled conditions, such as phytotron, are highly desirable for obtaining healthy donor material as well as for providing excellent flower buds to start shed-microspore culture in tropical conditions. Our preliminary studies in Wageningen, The Netherlands indicated that the hot pepper plants of 'Galaxy' and other pepper type grew well in phytotron conditions, and served as a better material for shed-microspore culture, resulting in high embryo yield (data not published). However, before we have such an expensive equipment for use in Indonesia, one should apply an efficient antibiotic treatment for certain periods only to avoid contamination.

The application of 100 µM colchicine treatment during the first week of culture at 9°C in darkness was highly efficient in increasing the DH plant production in shed-microspore culture. During this period, the first mitotic division of about 70% of microspores has already occurred (Supena *et al*., 2004). The efficiency of colchicine treatment appears to be not only due to the level of colchicine concentration, but also due to the optimal developmental stages and culture conditions. The optimal chromosome doubling was also associated with the duration of colchicine treatment and the culture temperatures (Saisingtong *et al*., 1996). The most effective period for the application of colchicine in anther or microspore culture was during the induction phase, i.e. the first microspore mitosis (Barnabás *et al*., 1991; Möllers *et al*., 1994; Saisingtong *et al*., 1996; Zhao *et al*., 1996; Antonie-Michard and Beckert 1997). It is known that colchicine efficiently arrests mitosis through microtubule depolymerization, resulting in homozygous doubled-haploid microspores (Barnabás *et al*., 1999). Thus, *in vitro* colchicine application has some advantages compared to the conventional *in vivo* application: 1) the procedure is simple and rapid, 2) it results in the occurrence of a high frequency of diploidization, and 3) low concentration can be used, avoiding the toxic effects. Hence, we suggest here that the application of 100 µM colchicine during the first week of culture should become an integral part of the refined shed-microspore culture protocol. The microtubule depolymerising herbicides, namely oryzalin, amiprophos-methyl, pronamide and trifluralin have also been reported to be less phytotoxic and cheaper, but were found to be similar to colchicine regarding their effects on chromosome doubling (Wan *et al*., 1991; Hansen and Andersen, 1996; Hansen *et al*., 1998).

The chloroplast number in guard cells of leaf stomata can be used to clearly distinguish between haploid and doubled haploid plants in hot pepper. Therefore, the counting of chloroplast number in guard cells of stomata can be considered as an effective indirect method for the analysis of ploidy levels in hot pepper plants of both large and curly types. Similarly, the chloroplast number had been reported as an effective indirect ploidy analysis in bell pepper types of *in vitro*-grown anther-derived plantlets (Qin and Rotino, 1995), and some other diploid and tetraploid pepper plants (Srivalli *et al*., 1995). Besides

being a relatively accurate and fast method, the counting of the chloroplast number in guard cells of stomata has some practical advantages in pepper, because there is no need of: a) high technical skill, as required for chromosome preparation and counting, and b) special and expensive equipment, such as flow cytometer. However, it should be noted that different growth conditions, such as light intensity, and developmental stages, such as the age and leaf position, may influence the number of chloroplasts. Therefore, it is highly desirable to use the plants grown under the same conditions and the leaves at the same developmental stage of growth for the analysis.

In conclusion, the results obtained in this study show that the combined treatment of antibiotics could solve bacterial contamination problems for shed-microspore culture under the tropical conditions of Indonesia. *In vitro* colchicine treatment could be integrated in the shed-microspore culture system to increase the doubled haploid production. The chloroplast counting in guard cells of stomata is simple, quick and an effective practical method to determine the ploidy level of regenerated plants in Indonesian hot pepper.

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Chapter 5

A new *Brassica napus* microspore embryogenesis system that mimics zygotic embryogenesis *ab initio*[*]

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Abstract

Inaccessibility of the zygotes and proembryos in angiosperms has hampered the efforts for studying early plant embryogenesis. Somatic and microspore-derived embryos are alternatives to perform molecular and biochemical studies, but they fail to be used in developmental studies due to the irregularity in early cell division patterns. In the present study, we report the establishment of a microspore embryogenesis system in *Brassica napus* that resembles zygotic embryo development *ab initio*. A mild heat-stress treatment of the microspores in culture induces this new procedure of embryo production. Notably, the microspores divide transversely several times first to form filamentous structures, of which the distal tip cells give rise to the embryo formation. The early division pattern of the embryos mimics that in zygotic embryogenesis. The lower part of the filamentous structure resembles the suspensor of a zygotic embryo. Using this system, we observed that the suspensor structure plays a role in the establishment of apical-basal polarity in the embryo. Multiple embryo formation was induced from the suspensor cell file when physical damage occurred in the suspensor. This *in vitro* system provides a powerful tool for studying the early stages of embryogenesis in plants.

Keywords: *Brassica napus*, microspore embryogenesis, polarity, suspensor, zygotic embryogenesis.

Introduction

In higher plants, embryogenesis is usually initiated from a single-cell zygote, the fusion product of a sperm and an egg cell. In some species, embryos can be produced naturally in the seed without fertilization through apomixis (Koltunow, 1993), or initiated directly from male and female gametes by androgenesis and gynogenesis, respectively (Bajaj, 1990; Ferrie *et al*., 1995). Further, due to the totipotency of plant cells, cells from different parts of the plant body can also be induced to undergo somatic embryogenesis. Although non-zygotic embryogenesis has many features in common with zygotic embryogenesis (Zimmerman, 1993), there are also some essential differences which are manifested during the initial stages of the embryo development (Dodeman *et al*., 1997; Mordhorst *et al*., 1997). The most striking difference is the lack of endosperm and suspensor tissues in non-zygotic embryos.

Different approaches have been followed to understand how embryogenesis is regulated in plants. Genetic studies to screen for embryo mutants, mainly in *Arabidopsis* and rice, have led to the identification of genes involved in the basic developmental processes, such as the initial asymmetric division of the zygote cell (Mayer *et al*., 1993), the first division in the apical daughter cell of the zygote (Hamann *et al*., 1999), the determination of apical-basal organization (Berleth and Jürgens, 1993; Lu *et al*., 1996), and the differentiation of shoot apical meristem (Barton and Poethig, 1993; Clark *et al*., 1993, 1995; Kayes and Clark, 1998; Laux *et al*., 1996) and root apical meristem (Di Laurenzio *et al*., 1996; Willemsen *et al*., 1998). Also, certain embryo mutants, such as *sus*, *twin* and *twn2* revealed information about embryo-suspensor interactions (Marsden and Meinke, 1985; Schwartz *et al*., 1994; Vernon and Meinke, 1994; Zhang and Somerville, 1997). Recently, molecular studies have provided additional information about the extra-embryonic suspensor tissue. A *MAPKK* kinase gene has been identified that likely confers the identity of the basal daughter cell produced after the first zygotic division, which forms the suspensor (Lukowitz *et al*., 2004). A so-called *WOX* gene family has been reported, whose members play a role in region-specific transcription in the initial embryo proper and suspensor immediately from the asymmetric division of the zygote onwards (Haecker *et al*., 2004). Physiological studies have been applied to isolated zygotic proembryos to elucidate the functions of different factors, such as auxin and auxin polar transport in pattern formation (Liu *et al*., 1993a and b; Fischer and Neuhaus, 1996). Although these isolated zygotic embryos are able to follow the pattern of zygotic embryos in the plant, the problem remains on how to dissect even younger embryos and zygotes for *in vitro* research.

Although tremendous advances in the understanding of plant embryogenesis have taken place, most genes involved in the process are still unknown. For instance, Meinke *et al*. (2003) estimate that about 2,500 to 3,750 genes are required to successfully complete the process of embryogenesis to produce a viable seed. With the use of cDNA microarray

analysis, it was indeed recently shown how a large number of genes are differentially expressed in the later stages of embryogenesis (Girke *et al*., 2000; Ruuska *et al*., 2002). Unfortunately, performing a similar genomics research on embryo initiation and early embryonic development is complicated by the fact that sufficient amount of experimental material is difficult to obtain (Dodeman *et al*., 1997; Gallois, 2001).

Microspore-derived embryos, produced from isolated microspore cultures, have been used as an efficient alternative to provide a sufficient amount of young embryo materials for biochemical analysis and modern genomics. In particular, microspore cultures of rapeseed (*Brassica napus* L.) and tobacco (*Nicotiana tabacum* L.) have evolved to become the model systems for this purpose (Telmer *et al*., 1993, 1995; Custers *et al*., 1994; Touraev *et al*., 1996). Normally with stress treatment on hormone-free media, numerous embryos are produced from microspores without going through an intermediate callus phase. Differential screenings using microspore cultures lead to the identification of interesting genes important for plant embryogenesis (Garrido *et al*., 1993; Boutilier *et al*., 1994; Zarsky *et al*., 1995; Kyo *et al*., 2003).

In our research group *B. napus* microspore culture has been used for studying the molecular mechanisms controlling embryo initiation and pattern formation for some years. During these studies, mRNAs and proteins specifically accompanying the induction of embryogenesis have been identified (Cordewener *et al*., 1995, 2000; Custers *et al*., 2001), and critical genes related to embryo induction and cell differentiation are elucidated (Boutilier *et al*., 2002; Fiers *et al*., 2004).

However, one drawback of using the microspore embryogenesis as a model for studying embryo development is that the developmental pattern at the early stage of embryogenesis shares a very little similarity with the zygotic one. Early zygotic embryogenesis in *Brassica napus* is characterized by a highly ordered cell division pattern (Tykarska, 1976), while the divisions in microspore embryogenesis are rather unorganised (Telmer *et al*., 1995; Yeung *et al*., 1996). Further, normal suspensor formation is absent in microspore embryogenesis, although occasionally a rudimentary suspensor-like structure is observed (Ilić-Grubor *et al*., 1998; Yeung, 2002). These morphological differences might be accompanied by gene expression differences, making the conventional microspore embryogenesis less suitable for molecular studies on early *in planta* embryo initiation.

The present paper describes a new procedure of *B. napus* microspore culture in which a high frequency of suspensor-bearing embryos are formed. The distinct cell lineage and organ patterning in these embryos resemble the route of sexual embryos of *B. napus*. This system, therefore, provides a unique starting point for molecular analysis of early embryo initiation processes *in vitro*.

Materials and methods

Growth conditions of the donor plants

Plants of *Brassica napus* L. cv. Topas line DH 4079 were grown year-round in a glasshouse at a 20/18°C day/night temperature regime. Additional light (Philips SON-T lamps) was given from mid September to the end of April, which also served to obtain a photoperiod of 16 h. At the beginning of bolting, the plants were transferred to a growth chamber at continuous 10 °C with 16 h illumination provided by 150 $\mu E.m^{-2}.s^{-1}$ HPI (Philips) light, and watered twice a week with N:P:K=15:15:18 soluble fertilizer. Inflorescences were harvested from plants kept at least two weeks under these conditions, after the first flowers had opened. Flower buds of 3.1-3.4 mm in length were selected and divided over three bud length classes (3.1-3.2, 3.2-3.3, and 3.3-3.4 mm), and from each class 15-25 buds were used for the isolation of microspores.

Microspore isolation and culture

Flower buds were sterilized for 10 min with 4°C 2% NaOCl + 0.05% (v/v) Tween-20, rinsed 3 times in 4°C sterile water for 1, 5 and 10 min, consecutively, and transferred to 50 ml disposable buckets containing 1.5 ml 4°C filter sterilized NLN-13 medium. This is a Nitsch and Nitsch medium as modified by Lichter (1982) with 13% (w/v) sucrose and without potato extract or growth regulators. The buds were gently squeezed for 5 to 10 sec with the back of the plunger of a disposable 50 ml syringe. The slurry was passed through two-layers of 44 μm nylon cloth, then bucket, plunger and cloth were rinsed with another 8.5 ml 4°C NLN-13 medium. The filtrate was centrifuged at 100g for 3 min in a refrigerated centrifuge at 4°C, and the pellet resuspended in fresh 4°C NLN-13 medium. After two additional washings, the microspores were resuspended at a density of 40,000 per ml in NLN-13 medium at room temperature. Aliquots of 3 ml microspore suspension were plated in 6 cm Petri dishes for culture. Developmental stages of the microspores at the start of culture was determined using 4',6-diamidino-2-phenylindole (DAPI) epifluorescence staining according to Custers *et al*. (1994).

For the induction of embryogenesis, the cultures were initially given high temperature treatments (28-34°C) for various periods of time in various Marius incubators, i.e. HTD drying-incubator, HTB culture incubator, or 85HTK minimum-maximum tissue culture incubator (Marius Instruments, Utrecht, The Netherlands), and thereafter kept at 25°C continuously. All the cultures were kept in darkness. To follow the early development of microspores in culture, cytological examination was conducted periodically during the first two weeks of culture, using DAPI staining and observations under epifluorescence microscope. Thereafter, progressive development in cultures was followed by observations under inverted microscope and stereomicroscope. Final data on embryo yield and their characteristic morphology during the process of embryogenesis were collected from the microspore cultures of the bud-length class that gave the best performance. Usually this was the middle bud-length class of 3.2-3.3 mm.

Results

Optimisation of the microspore culture for reproducible production of suspensor-bearing embryos

Microspore embryogenesis in *B. napus* occurs as a result of a developmental switch from gametophytic development to sporophytic development after a heat-stress treatment, commonly by culturing the isolated microspores at 32.5°C for two days or longer. The heat-stress treatment firstly leads to the formation of rather undifferentiated multicellular structure within the microspore exine walls (Telmer *et al*., 1995; Yeung *et al*., 1996). After release from the constraints of the exine walls, these cell clusters develop into globular proembryos (Fig. 1A). A close observation reveals two distinct regions are regularly present in the initial multicellular structure (Ilić-Grubor *et al*., 1998). The larger one gives rise to the embryo, whereas the smaller one consisting of a few cells forms an appendant structure, mostly quite irregular, and later found as a protuberance at the future radicle pole of the embryo (Fig. 1B). In addition to the embryos with irregularly shaped appendices, occasionally embryos with a long uniseriate suspensor-like structure were also found (Fig. 1C), indicating that zygotic-like embryogenesis could be achieved from isolated microspores. Notably, early in culture we found also microspores that gave rise to the formation of filamentous structures (Fig. 1D), which could be the precursor to form embryos with suspensors.

In order to increase the production of suspensor-bearing embryos, we attempted to optimize the culture conditions. Several parameters of the protocol, for instance, the medium composition and centrifugation force during microspore preparation were evaluated, but without further improvement. However, we found that a minimum periode of heat-stress treatment and a slightly decrease of the temperature from 32.5 to 32.0°C resulted in an evidently increased frequency of formation of embryos with suspensors. The heat-stress treatment at 32.0°C for a period of 8-12 h led to the production of more than two thirds of the embryos carrying a long suspensor (Fig. 2A). While increasing the duration from 8 h to continuous treatment at 32.0°C, the frequency of embryos with long suspensors decreased gradually and reached almost zero. We also observed that when the microspores were cultured at 32.0°C continuously, the percentage of embryos without any appendix was increased. The intermediate type of embryos with short appendant structures, either uniseriate or swollen and irregular, were found in all the treatments, but the frequencies of such embryos were significantly higher when the microspores were treated at 32.0°C for 24 or 48 h, as compared to shorter or longer duration treatments (Fig. 2B).

The treatment of microspores at 32.0°C for 8-12 h, however, appeared to be not the only condition for obtaining high frequencies of suspensor-bearing embryos. The accuracy of temperature also appeared to be a critical factor to obtain a high frequency of suspensor-bearing embryos. The high frequency of suspensor-bearing embryos could be obtained only

when the Marius 85HTK tissue culture incubator with a temperature regime of 32.0±0.2°C was used, whereas the Marius HTD and HTB incubators showed much higher temperature amplitudes (±0.8-1.2°C), and were found to be unsuitable for obtaining high frequencies of embryos with suspensors (data not shown).

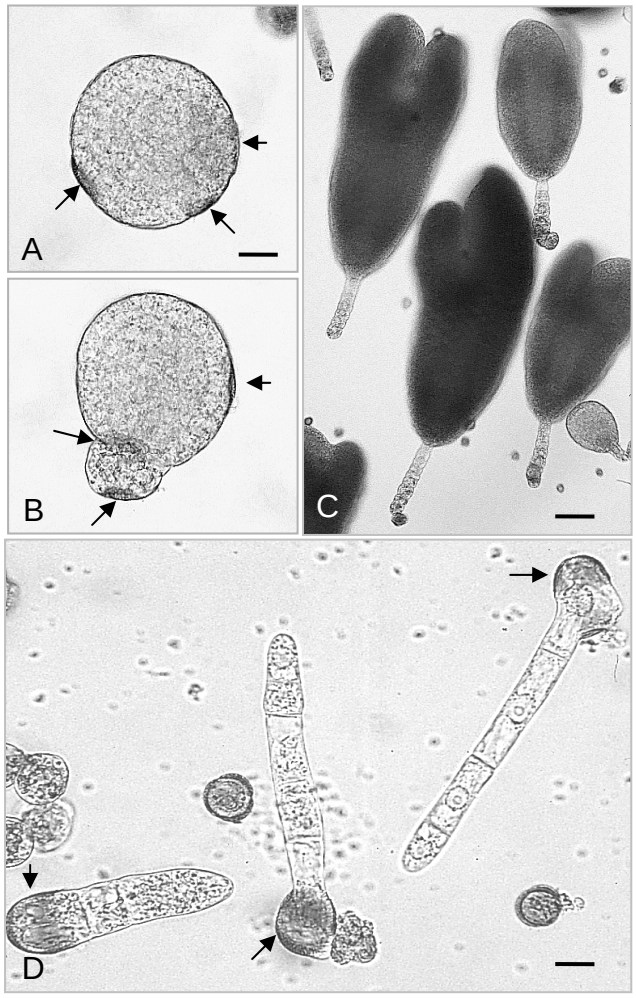


Figure 1. Various structures were detected in an embryogenic microspore culture of *Brassica napus*. **A** and **B**: Globular embryos covered with the remnants of the microspore exine wall (arrows), bearing or not bearing an appendix structure after 7 days of culture. **C**: Heart-shape embryos with suspensor-like structures after 16 days of culture. **D**: Filamentous structures emerging from the microspores (arrows), after 8 days of culture. **C** and **D** are from the later cultures that produced suspensor-bearing embryos at high frequencies. Bars = 20 µm for **A**, **B**, and **D**; 50 µm for **C**.

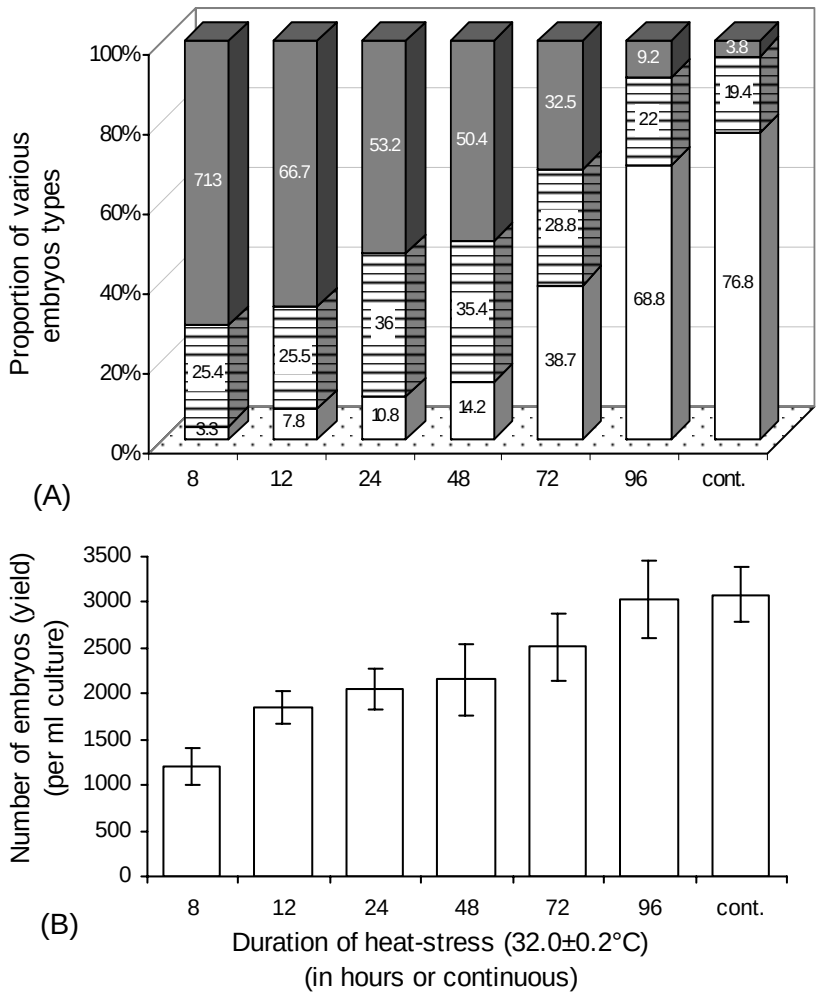


Figure 2. Effects of different periods of 32°±0.2°C heat-stress treatment in *Brassica napus* microspore culture on **A**: the frequencies of embryos with long suspensor (■), embryos with short suspensor (< 30µm unseriate or < 50µm irregular shaped appendix structures) (≡), or embryos without any appendix (□); and **B**: total embryo yield. Cultures started with a density of 40,000 microspores per ml. After the heat-stress treatment, cultures were kept at 25°C. For the estimation of total embryo yield, (in **B)**, embryos larger than 0.4 mm were counted after 14-16 days of culture, when the largest embryos had reached 3 mm already. Data are the means of five replicated experiments, each time of culture with three 6 cm Petri dishes per treatment, and 3 ml original microspore suspension per dish. Bar = ± standard error.

Sensitivity for heat-stress differs for the successive developmental stages of *B. napus* microspores incubated in culture. Usually, it is considered that particularly late unicellular microspores and early bicellular pollen are competent for the induction of embryogenesis (Pechan and Keller 1988; Telmer *et al.* 1992), but also more advanced-stage pollen appeared to be inducible, if a more severe heat treatment was applied (Binarova *et al.*, 1997). We studied the optimal composition of the microspore population for obtaining a high frequency of suspensor-bearing embryos upon heat treatment of 32.0±0.2°C. It was found that a well-responsive microspore population harvested from a sample of immature flower buds, should contain at least 60% of late unicellular microspores and not more than 20% of mid unicellular ones, whereas the percentage of early bicellular pollen should not exceed 40% of the population (data not shown).

In addition to a high frequency of embryo formation with suspensors, the total number of embryos produced is also important. Figure 2B presents the data on the total yield of embryos, as affected by different duration of 32.0±0.2°C heat-stress treatment. Although the embryo yield decreased with decreasing duration of heat-stress from continuous treatment to 8 h, optimal amounts of 1,000 to 1,200 suspensor-bearing embryos per ml were produced in cultures treated with heat-stress for 12-24 h. These are reasonable amounts, which are sufficient for different experimental purposes. The total embryo density could be enriched through sieving with a nylon filter (Fig. 3).

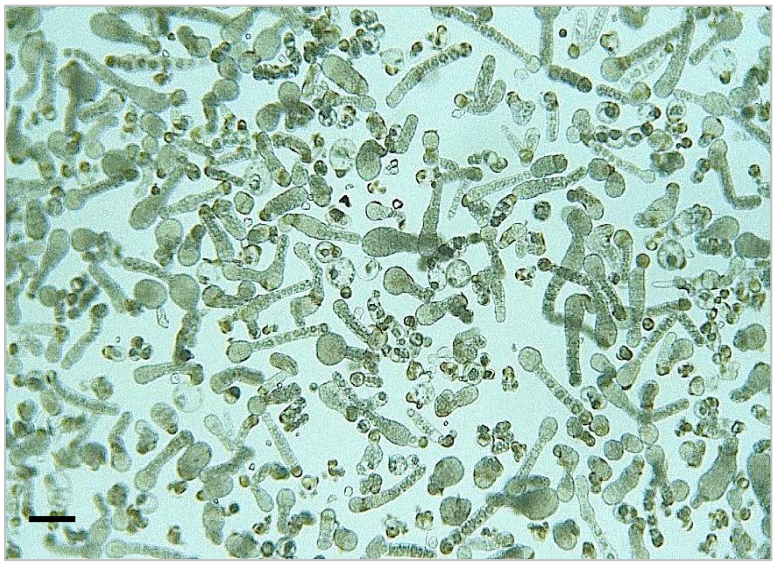


Figure **3.** A population of suspensor-bearing embryos produced from a *Brassica napus* microspore culture enriched through sieving after 10 days of culture. The microspores were treated at 32.0±0.2°C for 24 h and then transferred to 25°C, all being in darkness. Bar = 250 µm.

Cell tracking experiment suggests a zygotic-like microspore embryogenesis

In order to follow the development of the filamentous structures over time, we carried out cell tracking and time-lapse photography of individual microspores (Fig. 4). The filamentous structures appeared first to emerge from microspores at day 6 or 7 of culture (Fig. 4A). They were found to be single files of cells, either slender or more swollen (Fig. 4B, 4C and 1D), with one end connected to the microspores. At day 8 of culture (Fig. 4C), most of them have 3-8 cells in the files. Thereafter, the distal ends of the files, opposite to the remnant microspores, began to swell (Fig. 4D) to produce a globular body (Fig. 4E), while the number of cells in the files still increasing. The globular bodies eventually developed into early heart-shape embryos (Fig. 4F-I). Normally, the cell division in the files was ceased when the embryo reached the late globular stage. Thus, the cell tracking experiment demonstrated that embryos with a suspensor-like structure in these cultures were originated from pre-existing filamentous structures. Further, it showed that the course of the embryogenesis process strongly resembles that of the zygotic embryo development. In particular, the divisions in the filamentous structures mimicked those in suspensors of the zygotic situation.

Suspensor-bearing embryos make a slow start

Embryos without suspensor, most frequently produced through continuous culture at 32.0±0.2°C, showed their first sporophytic divisions after one to two days of culture. Four days later proembryos were formed, and at day 8, they have developed up to the globular to early heart-shape stage (Fig. 5A). In contrast, those single cell files from which embryos with suspensor will develop, appeared in culture only from day 6 onwards, and on day 8 these files consisted of 2-8 cells (Fig. 5B). At that time, embryogenesis from the distal end of the files was still not observed. One week later, when the cultures were 15-days-old, the embryos without suspensor had already reached the torpedo stage and measured 1-3 mm in length, whereas the suspensor-bearing embryos were still at the globular or heart-shape stage, with a diameter of 100 to 300 µm (suspensor not included for the size measurement). This large size difference, due to difference in timing, is probably the reason why embryos with long uniseriate suspensors have rarely been reported in conventional cultures in the literature (Pechan *et al*., 1991; Hause *et al*., 1994; Yeung *et al*., 1996; Straatman *et al*., 2000). Generally, the tiny suspensor-bearing embryos were difficult to detect among the well-developed embryos without suspensor unless particular attention was given.

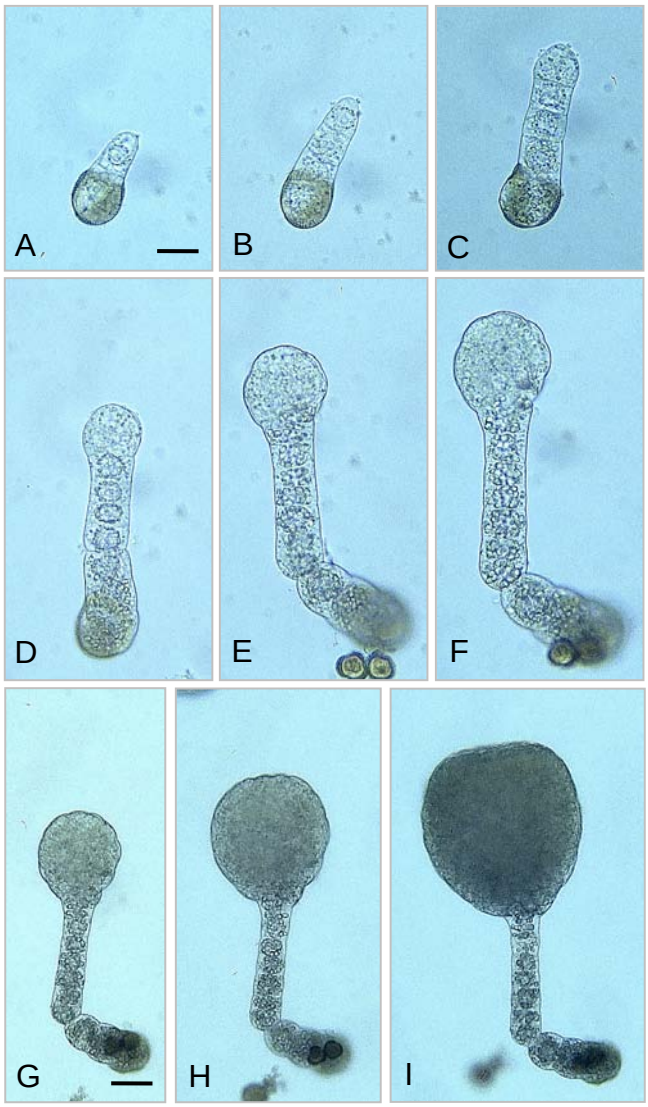


Figure 4. Representative time-lapse photographs showing the development of an embryo with a suspensor-like structure in *Brassica napus* microspore culture. **A**: A filamentous structure emerging from a microspore. **B** and **C**: Extended growth of the filamentous structure through transverse divisions. **D**: Swelling of the distal cell opposite to the microspore. **E-I**: Globular body developing into an early heart-shape embryo. **D** and **E** stages were accompanied with cell division in the filamentous structure; cell division ceased from **F** stage onwards. Photographs were taken at one day interval from day 6 to day 14 of culture. Bars = 30 µm **A-F**, and 40 µm for **G-I**.

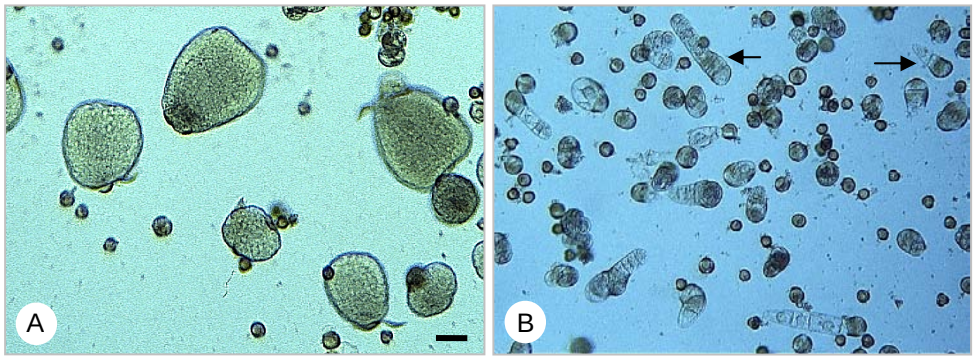


Figure 5*. A *Brassica napus* microspore culture, producing embryos without suspensors at day 8 of culture (**A**) compared with a culture of similar age with filamentous structures (arrows), which eventually will form embryos with suspensors (**B**). The culture **A** had been kept continuously at 32.0±0.2°C, while the culture **B** was treated with 32.0±0.2°C for 24 h, and then transferred to 25°C, where it was kept for 7 days. The filamentous structures consisted of 2-8 cells (arrows), while the globular to early heart-shape embryos had roughly 200-1,000 cells. Bar = 35 µm for **A** and **B**.
(***See appendix for color figure in page 131***)

The initial cell division pattern in suspensor-bearing embryos from microspores highly mimics that in zygotic embryos

In the new system of *B. napus* microspore embryogenesis, the distal ends of the single cell files gave rise to the development of embryos. In order to study the pattern of division that resulted in early embryo formation, samples were taken from cultures at regular intervals and observed with 4', 6-diamidino-2-phenylindole (DAPI) staining to visualize the nuclei (Fig. 6). It was seen that the filamentous structures consisted of a single row of cells, formed through transverse divisions (Fig. 6A and B). A change in this division behavior occurred when the distal tip cell underwent a longitudinal division (Fig. 6C), which further divided longitudinally giving rise to a 4-cell embryo proper. This change in division plane is critical, since it has been considered to be a landmark in zygotic embryogenesis of all crucifer species (Tykarska, 1976). The next in the embryo proper was the transverse division, producing 4 top cells and 4 bottom cells (Fig. 6D). Thereafter, another important division occurred, in which all 8 cells in the embryo proper underwent a periclinal division, to produce 8 protodermal cells and 8 inner cells (Fig. 6E). The cell just under the original tip cell of the linear file developed into a hypophysis-like cell with the shape of a lens (Fig. 6F and G). Ultimately, this cell got incorporated in the radicle zone of the embryo proper through a characteristic transverse and longitudinal division pattern (Fig. 6H).

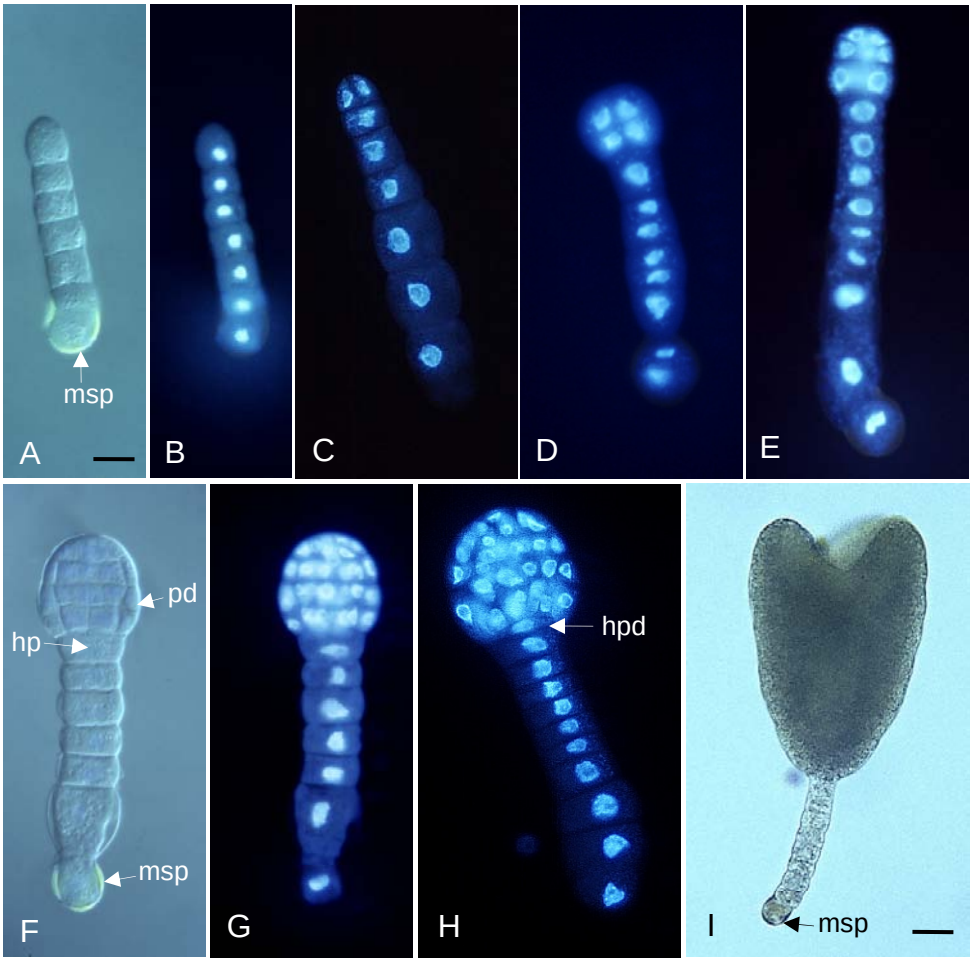


Figure 6*. Initial cell patterning during the development of embryos with a suspensor in *Brassica napus* microspore culture, observed with Nomarski optics (**A**, **F** and **I**) or examined under epifluorescence microscope after staining with DAPI (**B-E**, **G**, and **H**). **A** and **B**: Initial filamentous structure emerged from a microspore (**msp**); **C**: Longitudinal divisions in the distal tip cell leading to a 4-cell embryo proper; **D**: Octant stage proembryo (another four nuclei are present behind the four nuclei in focus); **E**: Periclinal cell divisions in the upper part of the embryo proper, forming the protoderm; **F** and **G**: Early globular stage embryo with protoderm (**pd**) and hypophysal cell (**hp**); **H**: Globular embryo with upper hypophysal daughter cell (**hpd**) incorporated in the radicle zone; **I**: Late heart-shape embryo with suspensor. Photographs taken from individuals after 8 (**A** and **B**), 9 (**C** and **D**), 10 (**E-G**), 11 (**H**), and 14 days (**I**) of culture. Bars = 30 µm for photographs **A-H**, and 55 µm for **I**.

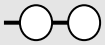
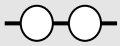
(***See appendix for color figure in page 131***)

Thus, these observations showed that the early steps of cell division in the new system of *B. napus* microspore embryogenesis had the same ordered division pattern as that occurring during the early zygotic embryogenesis. The continuation of the development brought about the formation of globular and heart-shape embryos with the attachment of a suspensor-like structure (Fig. 6I).

Position of the suspensors in full-grown embryos

After 14-16 days of culture, when the embryos were grown to late-heart and torpedo stages, we studied the position of the suspensor relative to the embryo proper. Normally embryos have the suspensor attached to the radicle pole, which represented the majority of the population (93% in Table 1). The opposite situation, in which the suspensor is attached to

Table 1. Frequencies of various embryo-suspensor combinations observed in a *Brassica napus* microspore culture pretreated at 32.0°±0.2°C for 24 h and then transferred to 25°C. Classification was based on the place, where proembryos were initiated in the linear cell files (proembryo stage), and on the later position of the suspensor (arms) relative to the apical and radical pole of the embryo (early heart-shape stage). More complex embryo-suspensor combination occurred at a frequency of < 0.1%.

Various embryo-suspensor combinations		Frequency (%)
Proembryo stage	Heart-shape stage	
		93.3
		2.1
		1.8
		1.7
		0.6
		0.2
		0.1
		0.1

the apical pole of an embryo, was never observed. Considering that the suspensor-like structures were formed slightly earlier than the embryos, this observation suggests that the suspensor may have a function in directing the apical-basal polarity in the embryo.

A stronger indication that the suspensor directs embryo apical-basal polarity was derived from embryos with two suspensors attached (Fig. 7). Such combinations, representing 4% of the population (Table 1), originated mainly when an embryo was initiated in the middle of a relatively long filamentous structure (Fig. 7A and B). We examined the course of this embryogenesis over time in order to see where the basal and apical zone would develop. One striking fact is that the suspensor was never observed on the shoot apex, even when two suspensors were attached to two opposite poles of a globular embryo. The apical dome failed to form the position where a suspensor was attached. However, the embryo seems able to adjust its polarity to form the shoot apices at the side, ending up with either one embryo with the second suspensor attached to the radicle, hypocotyls or the cotyledons, or two embryos with one suspensor each. Regularly, the apical dome formation was observed occurring perpendicular to the line along the two suspensor arms (Fig. 7C). In this case, eventually, an embryo developed with one suspensor arm connected to the radicle pole, while the other was connected at the side of the embryo (Fig. 7D). It was also seen that two apical poles developed (Fig. 7E), eventually resulting in a twin, wherein each embryo had a suspensor connected to its radicle pole (Fig. 7F). Remarkably, when embryogenesis was initiated in the middle of the suspensor structure, the whole process of embryogenesis took a long time; regularly it requires three weeks (from the first longitudinal division of the embryo proper) to reach the late-heart shape stage, which was only roughly a week when a normal embryo with one suspensor was formed (compare Fig. 6 and 7). From the images in Figure 7, one might deduce that the two suspensor arms counteract to another in directing progressive development of the embryo or embryos, causing the delay in the embryogenesis process.

Multiple embryo formation

In addition to the rather occasional development of embryos in the middle of suspensors in our cultures (in total 5% in Table 1), we found that the adventitious embryo formation along the suspensor structures could also be artificially induced in a solid medium. For easier observation of the developmental fate of the initial linear files, and for tracking experiments, we subcultured the isolated proembryos at the single-cell-file stage by transferring them from a liquid medium on top of a solid medium. Pretty soon under this condition, bending of most of the suspensors was observed, followed by the development of a string of several embryos along their entire length (Fig. 8). This was likely caused by the physical constraint imposed to the suspensor while it was cultured on a solid surface and continued elongation. This led to one or several breaks along the linear suspensor, and

consequently impeded the flow of nutrients and signals along the file, giving rise to physiologically-separated suspensor parts. In this way, each separated suspensor fragment would be able to induce its own embryo.

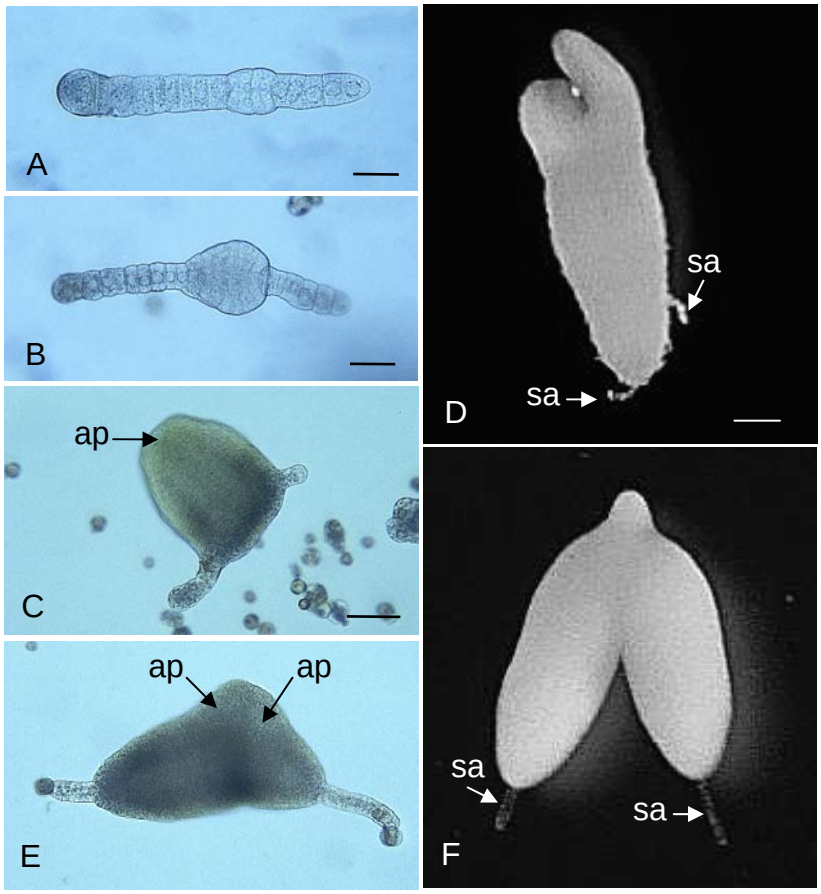


Figure 7. Developmental fate of embryo initials formed in the middle of relatively long linear cell files in *Brassica napus* microspore culture. **A**: Embryo initial in the middle of a file. **B**: Globular embryogenic structure in between two suspensor arms. **C**: Apical dome (**ap**) formation perpendicular to the line along the two suspensor arms. **D**: Embryo with one suspensor arm (**sa**) attached to the radicle pole and another to the hypocotyl area. **E**: Embryonic structure with two apical dome initials (**ap**). **F**: A twin embryo with apical domes under development and suspensor arms (**sa**) connected to both radicle poles. Photographs were taken after 9 (**A**), 11 (**B**), 16 (**C** and **E**), and 28 days (**D**, and **F**) of culture. Bars = 40 µm for **A**, 60 µm for **B**, 90 µm for **C** and **E**, and 110 µm for **D** and **F**.

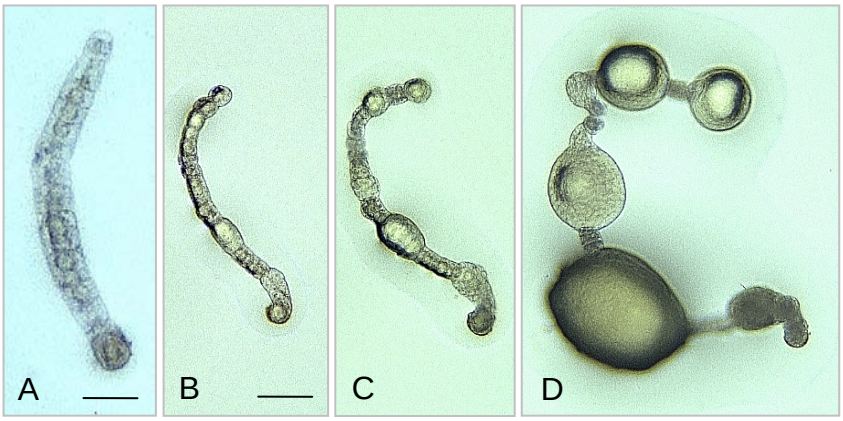


Figure 8. Developmental fate of a filamentous structure from *Brassica napus* microspore culture after subculture on solid medium. **A**: File with approximately 15 cells from a 10 days old culture, just upon subculture. **B**-**D**: Disappearance of the smooth cell file surface and formation of nodules along the file, which formed separate embryos. Five embryos with cotyledons were eventually obtained from this presented structure. Photographs were taken at 4 (**B**), 7 (**C**), and 10 days (**D**) after subculture on solid medium. Bars = 40 µm for **A**, and 100 µm for **B**-**D**.

Discussion

Microspore embryogenesis provides an efficient way to produce a large quantity of relatively synchronized embryos, and is therefore frequently used as a model system to study embryogenesis in plants (Touraev *et al*., 1996). *B. napus* microspore embryogenesis is considered to be one of the best models for this purpose (Custers *et al*., 1994; Telmer *et al*., 1993). However, as in other non-zygotic embryo culture systems, differences in the development of *B. napus* microspore embryos and zygotic embryos are routinely observed (Mordhorst *et al*., 1997; Thorpe and Stasolla, 2001), in particular as regard to the regularity of the initial cell divisions. In the zygotic embryos of *B. napus* the initial cell divisions are extremely defined (Tykarska, 1976), whereas microspore embryos initially develop through a series of unorganized cell divisions that only become ordered upon establishment of the protoderm. A second difference between microspore embryos and zygotic embryos is the partial or complete absence of a suspensor. In the present study, we established suitable culture conditions for the formation of embryos from *B. napus* microspores in a way that highly mimics the development of zygotic embryos. The new culture system produces high proportions of embryos attached to single cell files that look similar to normal suspensors.

Further, the regularity of the initial cell divisions in the embryo proper and subsequent early pattern formation were similar to that in zygotic embryogenesis.

The course of early cell divisions during initial zygotic embryo development in *B. napus* has been described in detail by Tykarska (1976). Upon fertilization, the zygote divides asymmetrically, forming a small apical cell and an elongated basal cell, from which the embryo proper and the suspensor will develop, respectively. The basal cell undergoes two transverse divisions before the apical cell shows its first characteristic longitudinal division. Thereafter, cell divisions continue in both the embryo proper and the suspensor. At the tetrad stage of the embryo proper, the suspensor consists of four, or occasionally of five cells. From here onwards up to the proembryo stage, two or three suspensor cells will undergo another division, and then suspensor divisions gradually get terminated. Finally, in the globular and heart-shape embryo stages, a number of 9-11 suspensor cells are observed (Tykarska, 1976; 1979). In the present new *B. napus* microspore system, the initial stages of the suspensor-bearing embryos showed a similar development as that occurring during early zygotic embryogenesis. Thus, this means that not only the cell division program in the embryo proper was similar to the one in zygotic embryos, but also the program of divisions in the suspensor exactly mimicked the zygotic counterpart. The most important factor for the improved culture system is the shorter heat-stress treatment at a slightly lower temperature, i.e. heat-stress treatment at 32.0°±0.2°C for 12-24 h and then transferred to 25°C.

Similarity of the newly established microspore embryogenesis system with zygotic embryogenesis was particularly evident when the microspore embryos had relatively short suspensors, consisting of 3-5 cells at the first division of the embryo proper. But also embryos with longer suspensors were found (6-12 cells, or even more, at the first division of the embryo proper), which have probably resulted from too long continuation of transverse cell divisions in this suspensor file. The beginning of the formation of the embryo proper was delayed in these embryos. One could propose that the extra long suspensor formation might be due to a weak embryonic signal in the embryo proper initial cell, or that the identity of this cell has not yet been determined. Alternatively, one might think that the suspensor has a function in determining the embryonic identity in the distal tip cell of the filamentous structure, and that when the suspensor fails in this, it causes the continuation of its own elangation. With respect to this, recently, Friml *et al.* (2003) discovered that in *Arabidopsis* zygotic embryos, polarity was established already from the earliest stages of embryogenesis onwards. Intensity of polarity, which was based on an auxin response gradient and found to be the highest in the zygote apical daughter cell and lower in the suspensor, appeared to increase with progress of developement up to the proembryo stage As proposed by the authors, this apical-basal auxin gradient specifies the identity of the apical embryo structures. On the analogy of the proposal by Friml *et al.* (2003), we assume that a similar auxin gradient is built up in the linear cell files in the

present study, and that it contributes to the embryonic identity specification of the tip cells in the files. Accordingly, the formation of extra long suspensors in our cultures will occur when the establishment of the auxin signal in the suspensor files is delayed or temporarily weak.

In addition to normal embryos with the suspensor attached to the radicle pole, we encountered in our new *B. napus* microspore embryogenesis system also characteristic aberrant embryo formations, in particular the embryo initiation in the middle of a suspensor, the formation of twin embryos, and the development of multiple embryos along the suspensor file. The particular experiments showed that the cells in a suspensor have embryonic developmental potential and can be induced to form embryos. These results are in broad outlines comparable with those from experiments on the *twin* and *twn2* mutants of *Arabidopsis*, showing that embryo proper defects allow the adventitious embryo formation from the suspensor cells (Vernon and Meinke, 1994; Zhang and Somerville, 1997). The common interpretation of these mutant phenotypes is, that the embryonic potential of the suspensor cells is always subordinate to the embryo proper, and that the suspensor cells can only express this potential after release from the repressive action by the latter (Meinke, 1995; Schrick and Laux, 2001). But we observed in our cultures some more details during the aberrant embryo formations, which were not fully covered by the concept of the *twin* and *twn2* mutants. For instance, in the case of embryo initiation from the middle of a suspensor file, we found that the entire embryogenic process took much longer than when a normal embryo with one suspensor was formed. It seemed that the two suspensor arms were counteracting to another in directing the progressive development of the embryo initial. The final result of this was so strange, that either a single embryo or a twin embryo was formed, whereas suspensors were always connected to the radicle poles and never to the apical ones. These observations as a whole are strong indication of an active role of the suspensor in guiding the apical-basal axis formation during the early stages of the embryo proper. Likely, the establishment of the early apical-basal polarity in the embryo will result from interaction between both the suspensor and the embryo proper.

The most exiting aberrant embryo formation in our cultures was seen when long suspensor files, after subculture on solid medium, gave rise to the formation of several embryos. We interpreted this multiple embryo formation to result from breakages in these suspensor files, leading to separated suspensor fragments, each of which with the ability to produce its own embryo. However, further experiments, e.g. laser cell-ablation, is needed to confirm this interpretation. Actually, our experiment is the first in giving experimental proof under *in vitro* conditions for the concept of the *twin* and *twn2* mutants as first proposed by Vernon and Meinke (1994), that during embryogenesis the zygotic apical cell lineage actively inhibits embryo formation from the basal cell lineage. As to the actual induction of the embryogenesis in the suspensor fragments, one could propose that cells that got damaged due to the breakage are causing adjacent cells to become embryogenic.

However, it might also be postulated that the suspensor fragment itself is specifying the embryonic identity of the cell at its the distal tip, likely through an auxin gradient signal built up in the short cell file. This idea fits well to the recent finding of Friml *et al.* (2003), that an apical-basal auxin gradient during initial zygotic embryogenesis specifies the identity of the apical embryo structures.

To conclude, we have presented here a refined *B. napus* microspore culture system, in which the embryo development resembles the zygotic embryogenesis. It is the first established system that can efficiently produce high frequencies of complete embryos, *ab initio* identical to zygotic embryos, from single differentiated cells in culture. The unique system will enable plant embryologists to establish future non-invasive technologies for studying early embryogenesis *in vitro*. Another application is that the procedure allows the production of sufficient quantities of young embryo material for transcriptomics and proteomics approaches during earliest stages of embryogenesis in plants. Exploiting this culture system in combination with many genomic tools available in *Arabidopsis* (full genome microarrays, sequenced genome, tagged mutants, etc) will allow fast progress in the molecular understanding of the events that control early embryo development in plants.

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Chapter 6

General Discussion

Ence Darmo Jaya Supena

Introduction

This thesis combines a series of investigations involving both applied and fundamental aspects of microspore embryogenesis. The main results of the applied part deal with the development and characterization of an efficient shed-microspore culture protocol for the production of doubled haploid plants in Indonesian hot pepper (*Capsicum annuum* L.), and its implementation under the local conditions of Indonesia. With regard to the more fundamental part of the thesis, a new microspore embryogenesis system has been developed in *Brassica napus* cv. Topas, and it is shown for the first time that completely normal embryos can be obtained from microspores in culture, which undergo exactly the same developmental pathway as that of zygotic embryos. These results will have potentially significant impact for practical application as well as for fundamental research on unraveling of early plant embryogenesis.

The results of the investigations are briefly discussed in the following sections with special emphasis on their relevance to theoretical and practical applications of microspore embryogenesis and their future prospects.

Development of shed-microspore culture in Indonesian hot pepper (*Capsicum annuum* L.)

Evaluation of the standard protocols for initiating a new protocol

Haploid and doubled haploid plants of Indonesian hot pepper (*C. annuum*) were produced in the present study through anther culture, directly isolated microspore culture and shed-microspore culture on the basis of protocols used previously in pepper (Dumas de Vaulx *et al*., 1982; Dolcet-Sanjuan *et al*., 1997) and tobacco (Johansson *et al*., 1982; Custers *et al*., 1999; Touraev and Heberle-Bors, 1999) (see Chapter 2). However, the efficiency of the directly isolated microspore culture was very low and appeared more genotype-dependent when compared to the two other methods. But, on the other hand, shed-microspore culture in a double layer system, as developed in the present study, has clearly outperformed the anther culture. Therefore, we focused our attention on the development and refinement of the shed-microspore culture protocol for Indonesian hot pepper in order to implement it under the local conditions of Bogor, Indonesia (Chapters 2-4).

All the 13 genotypes (open-pollinated varieties) and three DH lines of Indonesian hot pepper (Cayenne fruit type) showed embryogenesis after shed-microspore culture (Chapters 2-4). Explanation for these positive results can be that the shed-microspore culture system is either less genotype-dependent, or that the Indonesian hot pepper genotypes are highly responsive to such a shed-microspore culture system. On the contrary, for anther culture on solid medium, these genotypes were less responsive and showed genotype-dependency (Chapter 2). Previously, it has also been reported that the Cayenne type of pepper (more

spicy pepper) is less responsive than sweet-bell pepper in anther culture on solid medium (Munyon *et al.*, 1989; Qin and Rotino, 1993; Ltifi and Wenzel, 1994; Mitykó and Fári, 2001). Thus, it appears that the responsiveness of pepper for androgenesis depends not only on the pepper types or genotypes, but also on the culture systems used.

These experimental results suggest that for developing *in vitro* androgenesis protocols in a new crop species, it is desirable to assess the standard protocols using representative genotypes. To start with, the protocols in the same or closely related species can be tested using, for example, the three different culture systems, namely anther culture, shed-microspore culture and directly isolated microspore culture. Afterwards, attention can be focused on the development and improvement of the chosen protocol and culture system, using appropriate genotypes.

Development and improvement of the shed-microspore culture protocol

The shed-microspore culture protocol developed in the present study for Indonesian hot pepper was highly efficient and outperformed all the previously reported methods of haploid production in the entire pepper genus (Chapter 2). The production of normal-looking embryos and total embryo yield in the Indonesian hot pepper genotypes were further enhanced following the refinement of the protocol, i.e. the combined addition of zeatin and indole-3-acetic acid (IAA) to the culture medium after one or three weeks of culture, and by incubation at reduced temperature (18 or 21°C) after three weeks of culture (Chapter 3). Furthermore, the percentage of doubled haploid plants increased significantly after *in vitro* colchicine treatment during the first week of culture (Chapter 4).

In the protocol finally developed, we have combined several factors which were beneficial for the improvement of embryo yield and quality, and for increasing the frequency of DH plant production. Previous studies on *in vitro* haploid production systems, such as anther cultures, directly isolated microspore cultures and double-layer systems with pepper or other crops have shown that several factors can influence embryogenic ability and frequency of DH plants. We made a selection of the likely important factors from the literature, and we modified and combined them to achieve a haploid production procedure, that is highly efficient for the Indonesian hot pepper genotypes (see Table 1). Table 1 presents the main factors investigated in our new protocol besides their record in literature.

Table 1. Main factors in the shed-microspore culture procedure for Indonesian hot-pepper (*Capsicum annuum* L.), their specifications in the protocol, and related references on *in vitro* haploid production procedure with pepper or other crops

Main factors	Specification in the new protocol	Related references
Cold pretreatment of flower buds	Cold-stress (4ºC) pretreatment for one day	Sibi *et al*. (1979); Marrison *et al*. (1986); Gonzáles-Melendi *et al*. (1995)
Developmental stage of microspores	Microspore population with more than 60% in the late unicellular stage	Sibi *et al*. (1979); Gonzáles-Melendi *et al*. (1995); Barcaccia *et al*. (1999); Çiner and Tipirdamaz (2002)
Morphological markers for the optimal microspore stage	Anthers with purple tip covering 5-25% of the anther length	Sibi *et al*. (1979); Vagera and Havránek (1985); Regner (1994); Dolcet-Sanjuan *et al*. (1997)
Incubation temperature of the anthers	Cold-stress (9ºC) during the first week of culture	Johansson (1983); Dolcet-Sanjuan *et al*. (1997)
	28ºC during the second and third weeks of culture	Johansson (1983; Munyon *et al*. (1989); Dolcet-Sanjuan *et al*. (1997)
	21ºC or 18ºC from third week of culture onwards	Snider and Veilleux (1994); Rokka *et al*. (1996)
Light/dark	Continuously in dark	Mythili and Thomas (1995)
Medium composition	Nitsch and Nitsch medium: macro-, micro-elements, and vitamins	Nitsch and Nitsch (1969); Dolcet-Sanjuan *et al*. (1997)
	Maltose as carbon source	Dolcet-Sanjuan *et al*. (1997); Gemesné *et al*. (1998)
	Activated charcoal (1% w/v) in solid layer medium	Johansson (1983); Vagera and Havránex (1985); Dolcet-Sanjuan *et al*. (1997)
	Addition of zeatin (2.5 µM) + IAA (5.0 µM) after one or three weeks of culture	No closely related reference
In vitro colchicine treatment	Application of colchicine (100 µM) during the first week of culture to increase the frequency of DH plant production	Barnabás *et al*. (1991); Möllers *et al*. (1994); Saisingtong *et al*. (1996); Zhao *et al*. (1996)

Suspensor-bearing embryos in microspore culture of *Brassica napus* L.

Heat-stress controls the developmental fate of the microspores in culture

Brassica napus microspore culture is the most efficient microspore embryogenesis system to be used for fundamental research on early plant embryogenesis (Swanson *et al*., 1987; Pechan and Keller, 1988; Pechan *et al*., 1991; Boutilier *et al*., 1994, 2002; Cordewener *et al*., 2000; Custers *et al*., 2001). However, the embryo development in this system showed less similarity to zygotic embryogenesis, especially during the early developmental stages (Yeung *et al*., 1996; Custers *et al*., 2001; Yeung, 2002). The well-defined pattern of early cell divisions of zygotic embryos is absent during the early stages of microspore embryogenesis. Generally with *B. napus* microspore culture, a strong heat-stress treatment (i.e. 32.5ºC for two days or longer) is given in order to stop the gametophytic development of the microspores, and to change their fate into a sporophytic developmental program. The reiteration of sporophytic cell divisions lead to the formation of multicellular structures within the microspores, which appear rather unorganised (Fig. 1A). Due to the so-called "self-organising capacity" (Mordhorst *et al*., 1997), globular embryos and later heart-shape embryos develop from multicellular microspores. Only from their globular stage onward, microspore embryos show clear morphological resemblance with zygotic embryos. However, the microspore embryos do not bear the suspensors (Fig. 1A), which are the characteristic extra-embryonic appendices of zygotic embryos. Only rarely, an irregular clump of cells is attached to the radicle pole of microspore embryos, which is considered to be a suspensor-like structure.

In the present study, we developed a completely new procedure of *B. napus* microspore culture, in which embryogenesis from the microspores highly mimics the development of zygotic embryos including suspensor development (Chapter 5). From the first embryogenic cell division onwards, the patterning of divisions was highly similar to that in zygotic embryos (Fig. 1B). The main important factor to control this new system of microspore embryogenesis appeared to be a mild heat-stress treatment, viz. 32.0±0.2°C for 12-24 hours (Fig. 1B). Earlier, it was found that the temperature controls both gametophytic (Fig. 1C) and sporophytic development in microspore culture of *B. napus* (Custers *et al*., 1994). Now, this thesis showed in addition that a differential temperature regime controls the system of the sporophytic development, leading to embryogenesis. A strong heat-stress treatment induces the conventional pathway of microspore embryogenesis, with only partial development or complete absence of suspensor-like structure, whereas a mild heat-stress treatment gives rise to the formation of embryos that bear normal-looking suspensors. In the latter, new developmental pathway of microspore embryogenesis resembles the zygotic embryogenesis *ab initio*.

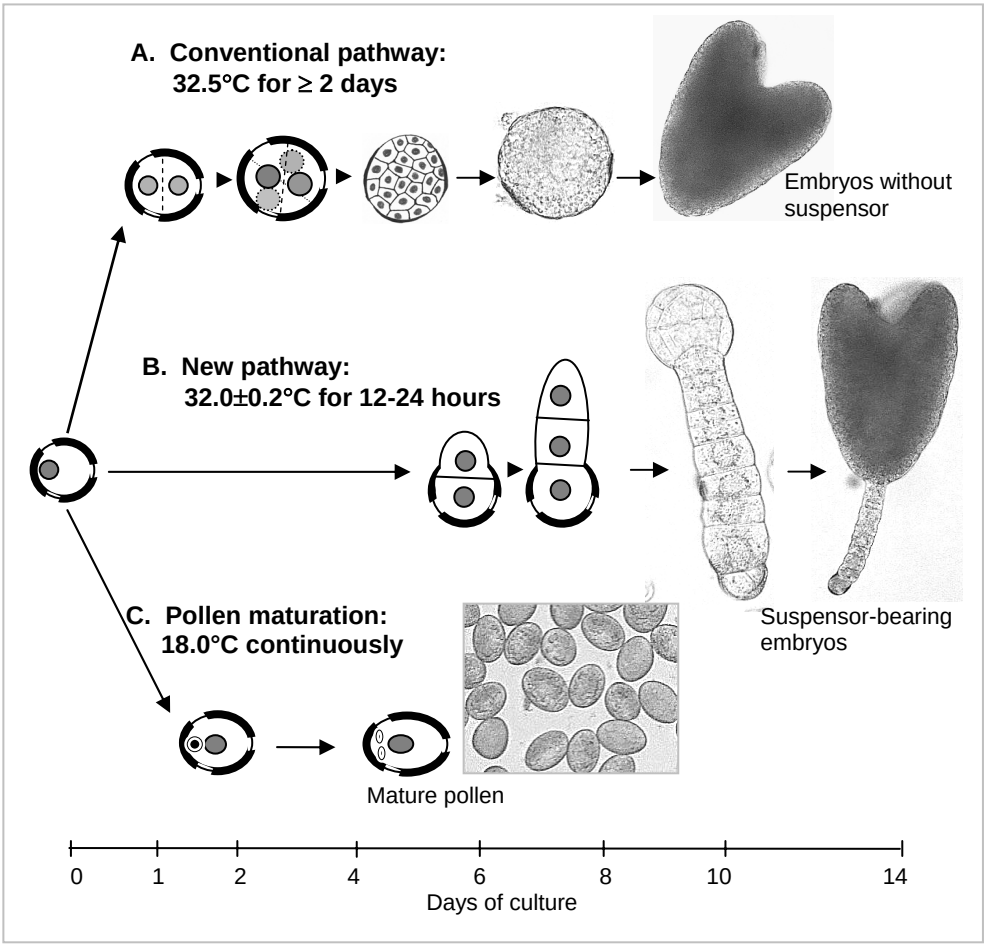

Figure 1. Temperature controls the developmental fate of *Brassica napus* cv. Topas isolated microspores in culture, viz. **A**: Conventional pathway of microspore embryogenesis as induced by a strong heat-stress treatment (32.5°C for two days or longer followed by incubation at 25°C) resulting in embryos without suspensor; **B**: New pathway of microspore embryogenesis as induced by a mild heat-stress treatment (32.0±0.2°C for 12-24 hours followed by incubation at 25°C) resulting in suspensor-bearing embryos; **C**: Gametophytic development resulting in pollen maturation when cultures are kept at low temperature of 18.0°C continuously.

Mild heat-stress induces the formation of suspensor-like structure

Most interesting finding in the new developmental pathway of microspore embryogenesis is that first single cell files emerged from the microspores in culture. These files in some cases look rather similar to the pollen tubes, but with respect to the diameter of the tube structure more than twice of that of the pollen tube. Further, they are also different from germinating pollen tubes, because the file of single cells have been formed through sporophytic cell divisions. This notable finding would suggest that formation of the suspensor-like structure in the new microspore embryogenesis procedure has resulted from the combined effects of gametophytic and sporophytic factors. Figure 2 shows a model for the formation of the suspensor-like structures from the microspores. The model supposes that the mild heat-stress treatment changed the developmental fate of the microspores only partially from a gametophytic to sporophytic program.

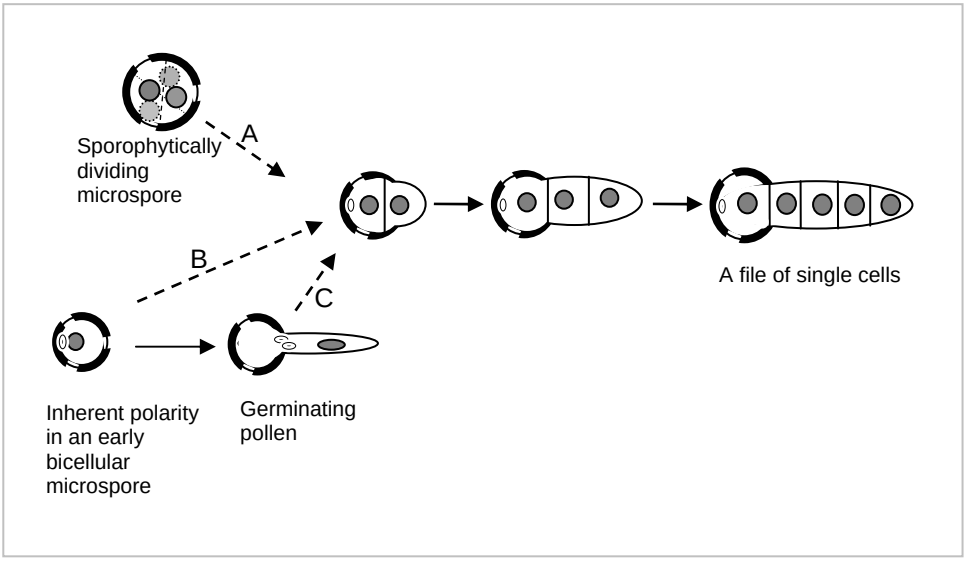

Figure 2. Theoretical model on the formation of a file of single cells from *Brassica napus* microspore culture pretreated with a mild heat-stress of 32±0.2°C for 12-24 h. Both sporophytic and gametophytic factors contribute to the development of single file cells, viz. **A**: ability of the microspores to undergo sporophytic divisions as induced by a mild heat-stress treatment; **B** and **C**: remnant of microspore or pollen characteristics, namely an inherent polarity and pollen tube formation capacity, respectively.

In microspores, an inherent polarity is established when the nucleus moves from the centre to the microspore wall (Ilić-Grubor *et al*., 1998; Twell *et al*., 1998). This nuclear migration occurs just prior to the first pollen mitosis, which is the most responsive stage for the induction of embryogenesis in microspore culture of *B. napus* (Binarova *et al*., 1997; Pechan and Keller, 1988; Telmer *et al*., 1992). We assume that this polarity is not fully disturbed after the mild heat-stress treatment. It imposes the first sporophytic division, which is also induced to follow a division plane perpendicular to the position of the nucleus in the late unicellular microspore or early bicellular stages. Following the first sporophytic divisions, it repeats the transverse divisions, resulting in the formation of a file of single cells (Fig. 2). Therefore, it seems that the initial inherent polarity in the microspores is carried in the single cell file. Apart from the fixed division plane during formation of the single cell files, the sporophytic divisions in the new microspore embryogenesis system differed considerably in the timing from those in the conventional culture system. In the new system the reiteration of sporophytic divisions started five days later than in the conventional system. We interpret this delay as caused by the mild heat-stress treatment.

Ultimate identity of suspensor and embryo proper

The results obtained in the present study clearly show that the new microspore embryogenesis system in most cases mimics the zygotic embryo development, and that the suspensor directs the embryo polarity. Further studies are, however, desirable, especially on the exact timing of the process when the initial cells become suspensor or embryo proper. In this regard, four possibilities can be mentioned:

(i) The timing is exactly similar to that of the zygotic situation, where the process takes place from early initiation onwards, i.e. the two cell stage is destined to become embryo proper and suspensor.
(ii) The single cell file has suspensor identity since the initiation, while the tip cell later becomes the embryo proper.
(iii) The reverse of the possibility-ii.
(iv) Both suspensor and embryo proper reveal ultimate identities during the development.

The morphological observations in the present study indicate the possibility-iv to be a more appropriate one. The ultimate identities of both embryo proper and suspensor do not appear to be destined from the first sporophytic division onwards, but they might probably occur during the later period of development.

Molecular, physiological and cytological analyses can shed light in this regard. *In situ* hybridisation using probes from specific genes expressed in the apical cell or basal daughter cells of the zygote after the first asymmetrical division of the zygote might facilitate the molecular evidence to address this question. In *Arabidopsis*, the *ATML1* gene (Lu *et al*., 1996), and recently the *WOX2* gene (Haecker *et al*., 2004) have been identified, which are

specifically expressed in the apical cell after the first asymmetrical division of the zygote. The *WOX8* gene is specifically expressed in the basal daughter cells of the zygote (Haecker *et al*., 2004). In addition, genes specifically expressed in the suspensor, such as *ASK*etha (Dornelas *et al*., 1999) and *G564* (Weterings *et al*., 2001) will be useful to prove the biological function of the filamentous structure to be a suspensor.

From the point of physiological studies, further research on polar auxin efflux and local auxin response would be very useful, because as shown by Friml *et al*. (2003), the first manifestation of an apical-basal axis in plants produces a basal cell that transports, and an apical cell that responds to the signal molecule, auxin. Also, cytological investigation of the development of microtubules and microfilaments (Hause *et al*., 1992, 1993; Simmonds and Keller, 1999) is important for increasing our insight in early embryogenesis development.

Future prospects

Two important new findings in the present study, which have potential for practical application as well as for further basic research, are: (i) an efficient shed-microspore culture protocol for Indonesian hot pepper (*C. annuum*), and (ii) a reproducible new microspore embryogenesis system in *B. napus*, which is highly similar to zygotic embryogenesis. Important future prospects of these findings are:

- The shed-microspore culture will allow a routine production of DH plants in Indonesian hot pepper, and consequently speed up the genetic improvement and breeding of the crop.
- The shed-microspore culture protocol is also potentially useful in other pepper types, such as sweet or bell pepper.
- The shed-microspore culture is an effective alternative system for research on embryo quality and the defective shoot meristem problem in tissue culture of pepper.
- The new system of *Brassica napus* microspore embryogenesis is a powerful tool for studying early plant embryogenesis at molecular, biochemical and cytological levels.
- Suspensor-bearing embryos in the new system will facilitate research on the dynamics of embryo-suspensor interactions.

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Summary

Hot pepper (*Capsicum annuum* L.) is the most important vegetable in Indonesia because of its economical value and cultivated area. However, it is still considered as a low input crop with relatively low yield. This is mainly due to the heavy losses caused by pests and diseases. Moreover, the local breeding programs are confined to the conventional methods. These methods are inefficient for the improvement of the crop with respect to polygenically controlled traits, such as resistance against diseases. Therefore, new technologies, such as haploid technology, are obviously necessary to speed up the breeding programs in Indonesia.

Haploid technology includes the regeneration of haploid embryos from gametes and the production of haploid and doubled haploid (DH) plants from them. This technique is the most rapid route to achieve in one step homozygosity (production of pure lines) instead of using seven generations. Embryo formation from microspores (the immature pollen) is nowadays the most efficient procedure for the production of DH plants. In addition to the practical application for breeding and crop improvement, microspore embryogenesis is also well-known as a model system for fundamental research on plant embryogenesis.

The primary goal of the research presented in this thesis was to combine both applied and fundamental research in microspore embryogenesis. The applied part deals with the development of an efficient protocol for the production of DH plants in various Indonesian hot pepper (*C. annuum*) genotypes, and thereafter to implement the technology under local conditions of Indonesia. The fundamental research is mainly concentrated on microspore embryogenesis in *Brassica napus* cv. Topas and the production of suspensor-bearing embryos that mimic zygotic embryos.

To establish an efficient DH plant production method for Indonesian hot pepper, several systems of anther and microspore culture were studied (Chapter 2). A shed-microspore culture protocol was developed which outperformed all the previously reported methods of haploid production in pepper. The critical factors of the protocol are: selection of flower buds with more than 50% late unicellular microspores, a one day 4°C pretreatment of the buds, followed by culture of the anthers in a double-layer medium system for one week at 9°C, and thereafter at 28°C in continuous darkness. The medium contained Nitsch components and 2% maltose, with activated charcoal in the solid under layer. All the ten genotypes of hot pepper tested, responded to this protocol. The best genotypes produced four to seven plants per original flower bud, which equal to 72-125 plants per 100 anthers.

The shed-microspore culture protocol is further improved in Chapter 3. The main emphasis was on quality of embryos produced, which so far was not satisfactory. The most important factors that contributed to the improvement of embryo quality, i.e. the production of normal embryos at high frequencies, include: a) the selection of anthers based on the

appearance of a purple tip area covering 5-25% length of the anther (≥60% of the microspores in the late unicellular stage), b) addition of 1% activated charcoal in the solid under layer medium, c) delayed enrichment with 2.5 µM zeatin and 5 µM indole-3-acetic acid in liquid upper layer medium after one or three weeks of culture, and d) the reduction of incubation temperature from 28°C to 21°C or 18°C after three weeks of culture. The improvement of embryo quality was possible also because of the use of doubled haploid lines as donor plants of the experimental anther material, and distribution of anthers at random over treatments. These two factors have clearly decreased the variability within a given treatment, and improved the statistical analysis of treatment effects.

The refined shed-microspore culture protocol is now suitable for implementation in the breeding programs of hot pepper. However, there were still some problems that hampered the implementation of the technology in Indonesia. Therefore, three crucial factors were evaluated and adapted to implement the protocol under the local conditions of Indonesia (Chapter 4). First, the application of a combination of the antibiotics timentin and rifampicin prevented bacterial contamination from the donor explants and eliminated the phytotoxic effects in culture, resulting in the production of embryos in all the three hot pepper genotypes tested under local conditions of Bogor in Indonesia. Second, *in vitro* application of colchicine (100 µM) during the first week of culture was highly effective in increasing the percentage of doubled haploid plants. Third, chloroplast counting in guard cells of leaf stomata was found to be a reliable, fast and easy method to determine the ploidy levels in various types of Indonesian hot pepper in the local laboratories.

With regard to the more fundamental research of the thesis, attention was focused on microspore embryogenesis in the model species *B. napus*, mainly aiming to establish a new procedure of embrogenesis from microspores, which highly mimics zygotic embryogenesis (Chapter 5). In angiosperm seeds, the inaccessibility of zygotes and initial embryos due to their small size and location in the very tip of the ovule, is a major obstacle for molecular and biochemical studies on embryo initiation and early embryonic development. Therefore, high quantity production of microspore embryos that fully resemble zygotic counterparts, might provide alternative experimental material for the molecular and biochemical research on early plant embryogenesis. In the present research, it was found that a mild heat-stress treatment (32.0±0.2°C for 12-24 hours) of the microspores in culture was highly essential for the production of suspensor-bearing embryos at a high frequency. The microspores first formed filamentous structures, of which the distal tip cell gave rise to embryo formation. Early pattern formation of these embryos was identical to that in zygotic embryogenesis. The suspensor structure was found to play a role in the establishment of apical-basal polarity in the embryo. The new pathway of *in vitro* microspore embryogenesis developed in this study, makes feasible the reproducible production of suspensor-bearing embryos on a large-scale, and could serve as a tool for a better unraveling of early embryogenesis in plants.

Further, the importance of new findings in the present study is highlighted and briefly discussed in Chapter 6. An efficient shed-microspore culture protocol developed for Indonesian hot pepper will be implemented for a routine application to produce doubled haploid plants. This will speed up hot pepper genetic improvement in breeding programs in Indonesia. The shed-microspore culture system is also potentially useful in other pepper types, and it can be used as a model system to study the embryo quality problem regarding the defective apical shoot formation in pepper during *in vitro* culture.

With regard to the reproducible new microspore embryogenesis system developed in *B. napus*, which highly mimics zygotic embryogenesis, it will enable to establish the future non-invasive technologies for studying early plant embryogenesis in living material. The new system will also allow to produce sufficient quantities of material for transcriptomics and proteomics approaches during the earliest stages of embryogenesis in plants. Further, the exploitation of the new *B. napus* culture system in combination with many of the tools of genomics available in *Arabidopsis* will allow a fast progress in the molecular understanding of the events that control early embryo development. In conclusion, the novelties from microspore embryogenesis in the present study have high potential for practical application in breeding programs of hot pepper as well as for further fundamental research in plant embryogenesis.

Samenvatting (Summary in Dutch)

Hete peper (*Capsicum annuum* L.) is het belangrijkste groentengewas in Indonesië zowel wat betreft economische waarde als geteeld areaal. Maar de teelt zelf is weinig geavanceerd en de totale opbrengst van het gewas is relatief laag. Dit laatste komt met name door de grote verliezen van product als gevolg van ziekten en plagen. Ook de veredeling van hete peper in Indonesië is nog erg conventioneel. Veredelingsprog-ramma's zijn weinig toegesneden op het verbeteren van eigenschappen in het gewas die polygeen zijn bepaald, zoals bijvoorbeeld ziektenresistenties. Om sneller resultaat te bereiken in veredelings-programma's zijn dan ook nieuwe technieken nodig, bijvoorbeeld het gebruik van haploïde planten.

Voor het gebruik van haploïden in de veredeling worden eerst haploïde embryo's geregenereerd uit de gameten en vervolgens wordt in de haploïde planten daaruit het chromosoomaantal verdubbeld. Dit levert verdubbelde haploïde planten op. Het is de snelste methode om homozygote planten en vervolgens zuivere lijnen te maken. Regeneratie van embryo's uit microsporen, het onrijpe stuifmeel van een plant, is tegenwoordig het meest efficiënte systeem voor het maken van verdubbelde haploïde planten. Toepassing ten behoeve van de veredeling is echter niet het enige belang van embryogenese uit microsporen, het proces zelf is namelijk ook ontwikkelingsbiologisch interessant en embryogenese vanuit microsporen is daarom een bekend modelsysteem voor het bestuderen van de embryogenese bij planten in het algemeen.

Doel van het onderzoek in dit proefschrift was om toegepast onderzoek en fundamenteel onderzoek aan embryogenese vanuit microsporen te combineren. Het toegepaste onderzoek was gericht op het ontwikkelen van een goed werkend systeem voor de productie van verdubbelde haploïde planten bij verschillende typen Indonesische hete pepers en om dit daarna ter plaatse te implementeren. Het fundamentele onderzoek werd uitgevoerd bij het modelgewas voor embryogenese vanuit microsporen, namelijk *Brassica napus*, en had tot doel vanuit de microsporen embryo's met suspensors te regenereren op dezelfde manier als bij de zygotische embryogenese.

Voor het ontwikkelen bij hete peper van een systeem voor productie van verdubbelde haploïde planten werden verschillende vormen van helmknopcultuur en microsporen-cultuur onderzocht (Hoofdstuk 2). Uiteindelijk werd een zogenaamd "microsporen weg-schiet-systeem" ontwikkeld, dat meer verdubbelde haploïden produceerde dan alle eerder in de literatuur gerapporteerde methoden bij pepers. Kritische factoren in het protocol zijn: selectie van bloemknoppen in het juiste stadium (met ≥ 50% laat unicellulaire microsporen), voorbehandeling van deze knoppen gedurende een dag bij 4°C, daarna incuberen van de helmknoppen op een dubbel-laags medium, eerst gedurende een week bij 9°C en vervolgens bij 28°C, steeds in donker. De vaste onderlaag van het medium bevat alle Nitsch bestanddelen en daaraan toegevoegd is 2% maltose en actieve houtskool. Tien

Indonesiche hete peper genotypen werden onderzocht en zij reageerden alle positief. De beste genotypen produceerden 4 tot 7 plantjes per bloemknop, wat neerkomt op een productie van 72-125 planten vanuit 100 helmknoppen.

Het “microsporen weg-schiet-systeem” werd vervolgens verbeterd in Hoofdstuk 3. Het ging hierbij met name om het verbeteren van de kwaliteit van de geproduceerde embryo’s, omdat die eerder nogal te kort schoot. Dit doel werd bereikt en de belang-rijkste factoren die daaraan bijdroegen waren: a) gebruiken van de hoeveelheid paarsviolet verkleuring van de helmknop als maat voor het ontwikkelingsstadium van de microsporen (5-25% van de lengte van de helmknop verkleurd betekende ≥ 60% laat unicellulaire microsporen), b) verhogen van het percentage actieve houtskool in de onderlaag van het medium tot 1%, c) verrijken van de bovenlaag van het medium met 2,5 μM zeatine en 5 μM indolazijnzuur na een of na drie weken cultuur, en d) verlagen van de temperatuur van de kweek van 28°C naar 21°C of 18°C na drie weken cultuur. Deze bevindingen die leidden tot de verbetering van de embryokwaliteit, werden met name mogelijk gemaakt door verdubbelde haploïde planten te gebruiken als donoren van de helmknoppen en door de helmknoppen at random te verdelen over de behan-delingen. Beide factoren hebben in belangrijke mate bijgedragen aan het verminderen van de variantie van de proefuitkomsten.

Het verbeterde “microsporen weg-schiet-systeem” was nu geschikt om te worden geïmplementeerd in verdelingsprogramma’s van hete peper. Maar er waren toch nog enkele problemen die daadwerkelijke implementatie in de Indonesische lokale omstan-digheden in de weg stonden. Drie cruciale zaken werden in Hoofdstuk 4 onderzocht. Allereerst het probleem dat in Indonesië nagenoeg alle donorplanten besmet zijn met bacteriën. Gevonden werd dat een combinatie van de antibiotica timentine en rifam-picine toegevoegd aan de kweek de bacteriën goed bestreed en bovendien in de cultuur geen fytotoxisch effect had. In Bogor in Indonesië werd dit systeem uitgeprobeerd, drie genotypen werden gebruikt en bij alle drie werden embryo’s verkregen. Het tweede probleem is dat het verdubbelen van het chromosoomaantal met behulp van colchicine bij planten op het veld heel moeilijk is. Als oplossing hiervoor bleek het toedienen van de colchicine (100 μM) aan de kweek gedurende de eerste week goed te voldoen. Dit was een effectieve manier om meteen al uit de kweek een voldoende hoog percentage verdubbelde haploïden verkrijgen. Als derde punt was een voor lokale Indonesische laboratoria eenvoudige methode voor het bepalen van het ploïdieniveau belangrijk. Tellen van het aantal chloroplasten in de huidmondjescellen van het blad bleek hiervoor als methode goed te voldoen.

Voor wat betreft het meer fundamentele onderzoek van het proefschrift bij de modelplant *B. napus* werd met name aandacht besteed aan het ontwikkelen van een systeem van embryogenese vanuit microsporen, waarbij de embryovorming exact op dezelfde manier verloopt als bij de zygotische embryogenese (Hoofdstuk 5). Als het lukt om op die manier in kweek grote aantallen embryo’s te maken, dan is dat uitstekend proefmateriaal voor het doen van biochemisch en moleculair onderzoek aan de vroege embryovorming bij

planten. De moeilijkheid bij de bedektzadigen planten is namelijk dat de zygoten en de initiële embryo's op de plant diep verborgen zitten in het uiterste puntje van de jonge zaadknoppen. En in elke zaadknop zit er slechts één. Zij kunnen daarom niet in voldoende grote aantallen worden verzameld voor het moleculair en biochemisch onderzoek. In het onderzoek in het proefschrift werd gevonden dat een milde warmtestress behandeling (32,0±0,2°C gedurende 12-24 uur) van de microsporen in cultuur resulteerde in de vorming van een hoog percentage embryo's met suspensors. Uit een microspore ontstond eerst een lintvormige structuur van cellen, waarvan de cel aan de top overging tot embryogenese. De vroege celdeling in de embryo's volgde precies het karakteristieke celdelingspatroon van de zygotische embryogenese. De lintvormige structuur vertoonde alle kenmerken van een suspensor. Aangetoond werd dat de suspensor een rol speelt bij het bepalen van de apicale-basale polariteit in het embryo. Dus met dit nieuwe systeem van embryovorming uit microsporen kunnen nu inderdaad grote aantallen embryo's worden gemaakt voor het daadwerkelijk bestuderen en ontrafelen van de vroege embryogenese bij planten.

Ter afsluiting van het proefschrift werden in Hoofdstuk 6 de nieuwe bevindingen bediscussieerd en werd hun relevantie besproken. Het goed werkende "microsporen weg-schiet-systeem" ontwikkeld voor hete peper zal in Indonesië worden geïmplementeerd voor routinematige productie van verdubbelde haploïde planten. Hierdoor zullen veredelingprogramma's voor genetische verbetering van hete peper worden versneld. Het "microsporen weg-schiet-systeem" is in potentie ook geschikt voor andere typen pepers. Verder kan het dienen als model om een algemeen probleem bij weefselkweek van pepers, namelijk slechte embryovorming als gevolg van niet goede aanleg van het apicale meristeem, door onderzoek op te lossen.

Het nieuwe, reproduceerbare systeem van embryogenese vanuit microsporen bij *B. napus*, dat precies hetzelfde verloopt als de zygotische embryogenese, maakt in de toekomst non-invasieve bestudering van vroege embryogenese in levend materiaal mogelijk. Proefmateriaal voor transcriptomics en proteomics onderzoek aan de vroege stadia van embryogenese bij planten kan nu in voldoende hoeveelheid worden aangemaakt. Verder zal het combineren van het nieuwe *B. napus* systeem met de vele genomics hulpmiddelen die beschikbaar zijn in *Arabidopsis* kunnen leiden tot snelle toename van kennis en inzicht in de moleculaire mechanismen die de ontwikkeling van een jong embryo sturen. Samengevat kunnen we stellen dat de nieuwe bevindingen uit het hier gepresenteerde onderzoek aan embryogenese vanuit microsporen een belangrijke bijdrage leveren aan zowel de hete peper veredeling als aan het fundamentele onderzoek aan embryogenese van planten.

Ringkasan (Summary in Indonesian)

Cabai (*Capsicum annuum* L.) merupakan tanaman sayuran terpenting di Indonesia ditinjau dari nilai ekonomis dan luas areal pertanamannya. Namun, budidaya cabai di Indonesia pada umumnya masih belum dilakukan secara intensif, sehingga produktivitasnya masih sangat rendah. Faktor utama penyebab rendahnya produktivitas adalah serangan hama dan penyakit. Sampai saat ini, program pemuliaan tanaman cabai di Indonesia masih menggunakan metoda konvensional, yang pada dasarnya tidak efisien untuk perbaikan tanaman ke arah sifat-sifat yang dikendalikan secara poligenik, seperti ketahanan terhadap penyakit. Oleh karenanya, teknologi baru seperti teknologi haploid ('haploid technology') sangat diperlukan untuk percepatan program pemuliaan tanaman di Indonesia.

Teknologi haploid mencakup regenerasi embrio dari sel gamet yang dilanjutkan dengan pembentukan tanaman haploid dan haploid ganda (disingkat 'HG') dari embrio tersebut. Teknik ini merupakan cara tercepat untuk mencapai homosigositas dalam satu generasi (memproduksi galur murni), sedangkan cara konvensional membutuhkan setidak-nya tujuh generasi. Pada saat ini, pembentukan embrio dari mikrospora (sel bakal serbuk sari) merupakan metoda yang paling efisien untuk menghasilkan tanaman HG. Selain untuk keperluan praktis dalam pemulian tanaman, embriogenesis dari mikrospora juga sudah biasa digunakan sebagai model dalam penelitian dasar embriogenesis pada tumbuhan.

Tujuan utama penelitian yang disajikan dalam disertasi ini adalah untuk melakukan penelitian dasar maupun terapan embriogenesis dari mikrospora. Penelitian terapan bertujuan untuk mengembangkan prosedur yang efisien dalam memproduksi tanaman HG dari beragam genotipe cabai lokal Indonesia (*C. annuum*) dan penerapannya pada kondisi lokal di Indonesia. Fokus utama penelitian dasar adalah untuk mempelajari embriogenesis dari mikrospora pada *Brassica napus* cv. Topas dan memproduksi embrio dengan suspensor yang sangat menyerupai embrio dari zigot.

Untuk mengembangkan metoda yang efisien dalam memproduksi tanaman HG cabai, beberapa prosedur kultur antera dan kultur mikrospora diuji dan dipelajari (Bab 2). Prosedur kultur sebar-mikrospora ('shed-microspore culture' yang selanjutnya disingkat 'KSM') dipilih dan dikembangkan dalam penelitian ini. Dari efisiensinya dalam memproduksi tanaman HG, prosedur KSM ini melampaui semua prosedur yang telah dilaporkan sebelumnya untuk cabai. Faktor dan tahapan utama dari prosedur ini meliputi: seleksi kuncup bunga yang mengandung ≥50% mikrospora pada fase satu-sel tahap akhir, praperlakuan kuncup bunga pada suhu 4°C selama satu hari, dilanjutkan dengan kultur antera pada sistem media dua-lapis (media cair di atas media padat) selama satu minggu pada suhu 9°C, berikutnya kultur diinkubasikan pada suhu 28°C dan selalu dalam kondisi gelap. Media yang digunakan mengandung unsur-unsur dari media Nitsch dan 2% maltosa, dan hanya untuk media padat ditambahkan juga arang aktif. Semua cabai lokal Indonesia yang diuji (10 genotipe) memberikan respon positif terhadap prosedur KSM ini. Genotipe

yang memberikan respon terbaik dapat menghasilkan 4-7 tanaman per kuncup bunga atau setara dengan 72-125 tanaman per 100 antera.

Prosedur KSM, lebih lanjut ditingkatkan lagi efisiensinya dalam Bab 3. Perhatian utama adalah untuk memperbaiki kualitas embrio yang dihasilkan melalui peningkatan frekuensi embrio yang normal, yang sejauh ini masih kurang memuaskan. Faktor utama yang memberikan sumbangan dalam perbaikan kualitas embrio meliputi: a) seleksi antera berdasarkan kriteria warna ungu pada ujung antera yang mencakup 5-25% panjang antera (≥60% mikrospora pada fase satu-sel tahap akhir), b) penambahan 1% arang aktif pada media padat, c) penambahan 2.5 µM zeatin dan 5 µM IAA pada media cair setelah satu atau tiga minggu dari kultur, dan d) penurunan suhu inkubasi dari 28°C menjadi 21°C atau 18°C setelah tiga minggu dari kultur. Perbaikan kualitas embrio ini dapat tercapai dikarenakan juga penggunaan galur HG sebagai tanaman sumber eksplan dan penyebaran antera secara acak terhadap semua perlakuan. Kedua faktor ini secara nyata dapat menurunkan keragaman dalam perlakuan dan memperbaiki analisa statistik pengaruh faktor perlakuan.

Prosedur KSM yang telah diperbaiki sangat layak dan siap untuk digunakan dalam program pemuliaan cabai. Sayangnya masih ada beberapa kendala yang menghambat penerapan teknologi ini di Indonesia. Untuk ini, tiga faktor penting dievaluasi dan diadaptasikan untuk penerapan teknik ini pada kondisi lokal di Indonesia (Bab 4). Pertama, penggunaan kombinasi antibiotik timentin dan rifampicin mampu mengatasi masalah kontaminasi yang diakibatkan bakteri dari sumber eksplan dan juga mengurangi atau bahkan menghilangkan pengaruh negatif antibiotik pada kultur, sehingga dapat dihasilkan embrio dari tiga genotipe cabai yang diuji pada kondisi lokal di Bogor, Indonesia. Kedua, perlakuan kolkisin (100 µM) selama satu minggu pertama dalam kultur *in vitro* sangat efektif dalam meningkatkan frekuensi embrio dan tanaman HG yang diperoleh. Ketiga, penghitungan jumlah kloroplas pada sel penjaga stomata daun merupakan metoda yang layak, cepat dan mudah digunakan untuk menentukan jumlah ploidi tanaman cabai pada kondisi umum laboratorium di Indonesia.

Untuk bagian penelitian dasar dari disertasi ini, embriogenesis dari mikrospora pada tanaman model *B. napus* menjadi perhatian utama dengan tujuan untuk mengembangkan prosedur baru embriogenesis dari mikrospora yang sangat mirip dengan embriogenesis dari zigot (Bab 5). Sulitnya mengisolasi zigot dan embrio pada tahap dini dari biji angiospermae, yang disebabkan ukurannya yang sangat kecil dan letaknya dalam ovul, merupakan kendala utama untuk mempelajari perkembangan tahap awal embriogenesis secara molekular maupun biokimia. Oleh karenanya, memproduksi embrio dari mikrospora yang sangat mirip dengan embrio dari zigot merupakan alternatif guna penyediaan bahan untuk penelitian tersebut. Dari hasil penelitian ini, ditemukan bahwa perlakuan cekaman-panas (‘heat-stress’) pada takaran sedang (32.0±0.2°C selama 12-24 jam) terhadap kultur mikrospora *B. napus* merupakan faktor penentu untuk memproduksi embrio dengan

suspensor dalam frekuensi yang tinggi. Mikrospora dalam kultur, pertama-tama membentuk struktur semacam filamen, kemudian sel pada bagian ujung dari struktur ini, yang berlawanan dengan asal sel mikrospora, akan tumbuh dan berkembang menjadi embrio. Mulai dari tahap awal, pola pembelahan sel pada proses perkembangan dan pertumbuhan embrio dari mikrospora ini sama dengan embriogenesis dari zigot. Struktur suspensor berperan dalam menentukan arah polaritas bakal pucuk dan akar dari embrio. Dengan sistem embriogenesis yang baru dikembangkan ini, sangat dimungkinkan produksi embrio dengan suspensor dalam jumlah besar dan dapat diulang, sehingga dapat dijadikan alat untuk mempelajari dan memahami lebih baik tahapan awal embriogenesis pada tumbuhan.

Lebih lanjut, penemuan baru dalam disertasi ini dibahas secara singkat tetapi menyeluruh pada Bab 6. Prosedur KSM untuk tanaman cabai yang telah dikembangkan dan terbukti efisien, akan segera diterapkan dan digunakan untuk memproduksi tanaman HG secara rutin guna percepatan perbaikan genetik dan pemuliaan cabai di Indonesia. Teknik ini sangat berpotensi untuk diterapkan pada jenis cabai lain seperti paprika, dan juga dapat dimanfaatkan sebagai wahana untuk mempelajari masalah kualitas embrio sehubungan dengan kegagalan pembentukan meristem pucuk pada kultur *in vitro* cabai.

Berkenaan dengan sistem embriogenesis dari mikrospora *B. napus* yang sangat menyerupai embriogenesis dari zigot, pengembangan teknologi *in vitro* lebih lanjut guna mempelajari pertumbuhan dan perkembangan embrio secara dini dalam keadaan hidup menjadi sangat dimungkinkan. Sistem baru ini, dapat juga menyediakan bahan dalam jumlah yang cukup untuk keperluan penelitian tahap awal embriogenesis melalui pendekatan transkriptomik dan proteomik. Lebih lanjut, pemanfaatan sistem baru yang dikombinasikan dengan perkembangan teknologi genomik yang tersedia pada tanaman model *Arabidopsis* akan memungkinkan percepatan pemahaman mengenai apa yang terjadi dan yang mengendalikan proses awal embriogenesis. Sebagai kesimpulan, inovasi dari hasil mempelajari embriogenesis dari mikrospora yang disajikan dalam disertasi ini, sangat berpotensi untuk dimanfaatan secara praktis dalam pemuliaan cabai maupun untuk penelitian dasar lebih lanjut tentang embriogenesis pada tumbuhan.

Curriculum Vitae

Ence Darmo Jaya Supena was born on October 2, just before the discovery of embryos from anther culture was published in 1964, in Karawang, 'the rice-barn' of West Java, Indonesia. He has completed his elementary school and middle school in a small countryside village, and then the secondary high school at SMA 2 Karawang in 1983. In the same year, he continued his studies at Bogor Agricultural University (IPB), Bogor for undergraduate program with a major in Biology and minor in Botany, and completed his studies in 1987.

In 1987, he joined as an assistant researcher in the Research Center for Biotechnology (RCB), IPB. Afterwards, in 1989 he got a permanent job at the Department of Biology, FMIPA-IPB. But, he still continued also his research work at RCB-IPB. In 1993, he obtained the MSc degree from IPB with the topic of his thesis "Soybean interspecific hybridization". During the tenure of his thesis work, he learned some related techniques at the Department of Crop Sciences, University of Illinois at Urbana-Champaign, IL-USA, where he stayed for six months in 1991. After his MSc thesis work, he continued his research on the use of the *in vitro* techniques and mutagenesis to speed up plant breeding programs under local conditions of Indonesia. From 1996-1999, he was awarded with a grant from "Competitive Grant of Higher Education" to start a research project in hot pepper.

In 2000, he came to Wageningen with the fellowship from QUE project for the overseas Ph.D. program. In July 2000, he got registered as a Ph.D. student at the Graduate School of Experimental Plant Sciences, Wageningen University with a research project on haploid technology in hot pepper. This research is a part of the Biotechnology Research Indonesia-Netherlands (BIORIN) project funded by KNAW, The Netherlands, as a collaboration project between RCB-IPB and Plant Research International, Wageningen-UR.

After obtaining his Ph.D. degree, he will return to Indonesia to continue his tasks at the Department of Biology-IPB and RCB-IPB, Bogor, Indonesia. Besides, he will try to transfer his research experience and knowledge gained in haploid technology to students at IPB. One of his dreams has been to develop and implement this technology to speed up local breeding programs of some important crops in Indonesia. Therefore, he would like to continue a close collaboration with experts from leading laboratories and institutes, such as Plant Research International, Wageningen-UR.

I have just begun; the success is yet to come.

***E-mail address*: e-darmo@indo.net.id**

List of related publications

Publication in scientific journals

1. E.D.J. Supena, S. Suharsono, E. Jacobsen & J.B.M. Custers (2004) Successful development of a shed-microspore culture protocol for doubled haploid in Indonesia hot pepper (*Capsicum annuum* L.). Plant Cell Reports (Accepted)
2. E.D.J. Supena, C.M. Liu, E. Jacobsen & J.B.M. Custers. A new *Brassica napus* microspore embryogenesis system that mimics zygotic embryogenesis *ab initio*. (2004, submitted for publication in the Plant Journal: Technical Advance)
3. E.D.J. Supena, Muswita, S. Suharsono & J.B.M. Custers. Evaluation of crucial factors for implementing shed-microspore culture of hot pepper (*Capsicum annuum* L.) in local conditions of Indonesia. (2004, submitted for publication in Scientia Horticulturae)
4. E.D.J. Supena & J.B.M. Custers. Fine-tuning of shed-microspore culture to improve embryo quality in Indonesian hot pepper (*Capsicum annuum* L.). (2004, submitted for publication in Plant Cell Tissue and Organ Culture)

Publications in proceedings

1. E.D.J. Supena & J.B.M. Custers (2001) Introducing haploid technology to accelerate hot pepper research and breeding in Indonesia. Proceedings of Seminar-Workshop: "Survival from the crisis, Indonesia agricultural community empowerment", Wageningen, 18 October 2000, p.62.
2. E.D.J. Supena & Jan B.M. Custers (2002) Establishment of haploid technology to improve hot pepper breeding efficiency in Indonesia. Proceedings of the International 7th Indonesian Student's Scientific Meeting, ISSM 2002. Berlin, October 4-6, 2002, pp.69-71.

Publications in book of abstracts (***congress/seminar/workshop***)

1. E.D.J. Supena & J.B.M. Custers (2002) Microspore culture in local Indonesian hot pepper accessions (2002) Book of abstracts at the 10th IAPTC&B Congress "Plant Biotechnology 2002 and Beyond", in Orlando-Florida, USA, June 23-28, 2002, p.119-A.
2. J.B.M. Custers, E.D.J. Supena & A.H.M. van der Geest (2002) Model plants pave the way to haploid technology; microspore embryogenesis in ornamentals. Book of abstracts at the 10th IAPTC&B Congress "Plant Biotechnology 2002 and Beyond", in Orlando-Florida, USA, June 23-28, 2002, p.11 Addendum Booklet.

3. E.D.J. Supena, C.M. Liu & Jan Custers (2003) *Brassica napus* microspore culture as biological model for studying suspensor function. Book of abstracts at Workshop: "Embryogenesis and development regulation in plants", Torino, Italy, March 6-7, 2003, pp.14-17.
4. R. Joosen, E.D.J. Supena, J. Custers & K. Boutilier (2003) Expression profiling of *Brassica napus* microspore embryo culture. Book of abstracts at Workshop: "Embryogenesis and development regulation in plants", Torino, Italy, March 6-7, 2003, pp.10-11.
5. E.D.J. Supena, C.M. Liu, E. Jacobsen & J.B.M. Custers (2003) A new *Brassica napus* microspore embryogenesis procedure that fully mimics zygotic embryogenesis. Book of abstracts at the IX International Conference on Plant Embryology, Brno, Czech Republic, September 1-3, 2003, p. 50.
6. E.D.J. Supena, C.M. Liu, E. Jacobsen & J.B.M. Custers (2003) A new *Brassica napus* microspore embryogenesis procedure that fully mimicking zygotic embryogenesis. Book of abstracts at the Dutch-Chinese Life Science Forum, Wageningen, October 12, 2003, p.35.
7. E.D.J. Supena, S. Suharsono, E. Jacobsen & J.B.M. Custers (2003) Efficient shed-microspore culture for haploid plant production in Indonesian hot pepper (*Capsicum annuum* L.) types. Book of abstracts at the Dutch-Chinese Life Science Forum, Wageningen, October 12, 2003, p.36.
8. R. Joosen, E.D.J. Supena, Jan Custers & K. Boutilier (2004) Transcriptome analysis of early embryo development using *Brassica* microspore-derived embryo cultures. Book of abstracts at the 9th International Symposium on Plant Seeds: "Seeds in the –omics Era". Gatersleben, Germany, May 15-19, 2004, p.77.
9. R. Joosen, E.D.J. Supena, J. Cordewener, O. Vost, J. Custers, T. America & K. Boutilier (2004) Functional genomic of gametic embryogenesis: transcriptome and proteome analysis of *Brassica* microspore-derived embryo cultures. Book of abstracts at COST 851 WG3 Workshop: "Gametic cells and molecular breeding for crop improvement". Tulln, Austria, September 7, 2004, p.5.

Training and Supervision Plan

The Graduate School of Experimental Plant Sciences

1. Preliminary theoretical study and regular research discussions

a. A450-203: Recombinant DNA and Genetic Modification.
   A700-207: Botanical Somatic Cell Genetics.
   A450-205: Molecular Plant Cell Biology.
b. Regular research discussion at Cluster Plant Reproduction, and research and thematic meeting at Business unit Bioscience, Plant Research International (PRI).
c. Attendance at internal colloquia at PRI and semi-annual research meeting and annual workshop of Biotechnology Research Indonesia-Netherlands (BIORIN).

2. Participation in the EPS Ph.D. Student Days

a. Poster presentation: "Shed-microspore culture in Indonesian hot pepper accessions", Utrecht University, March 27, 2003.
b. Poster presentation: "Transcriptome profiling of early embryo development using *Brassica* microspore derived embryo culture". Free University, Amsterdam, June 3, 2004.

3. Attendance at seminar series organized by the Graduate School EPS

a. Selected topics at "Seminar series frontiers in plant development".
b. Selected topics at "Flying seminars".

4. Participation in Spring/Summer/Autumn/Winter schools

a. AFLP in Plant Systematics and Breeding, Wageningen, May 16-18, 2001.
b. Environmental signalling: *Arabidopsis* as model, Utrecht, August 27-29, 2001.
c. Bioinformation Technology-1, Wageningen, December 8-16, 2004.

5. Participation in international congress or symposium

a. Oral presentation at the IX International Conference on Plant Embryology, Brno, Czech Republic, September 1-3, 2003: "A new *Brassica napus* microspore embryogenesis procedure that fully mimics zygotic embryogenesis".
b. Oral presentation at the 7th International Indonesian Student's Scientific Meeting, ISSM 2002, Berlin, Germany, October 4-6, 2002: "Establishment of haploid technology to improve hot pepper breeding efficiency in Indonesia".
c. Poster presentation at the 10th International Congress of Plant Tissue Culture & Biotechnology, IAPTC&B, Orlando-Florida, USA, June 23-28 2002: "Microspore embryogenesis in local Indonesian hot pepper accessions".

6. Participation in other post doctoral education

Techniques for writing and presenting a scientific paper, Wageningen, June 29 – July 2, 2004.

Appendix

(Color figures)

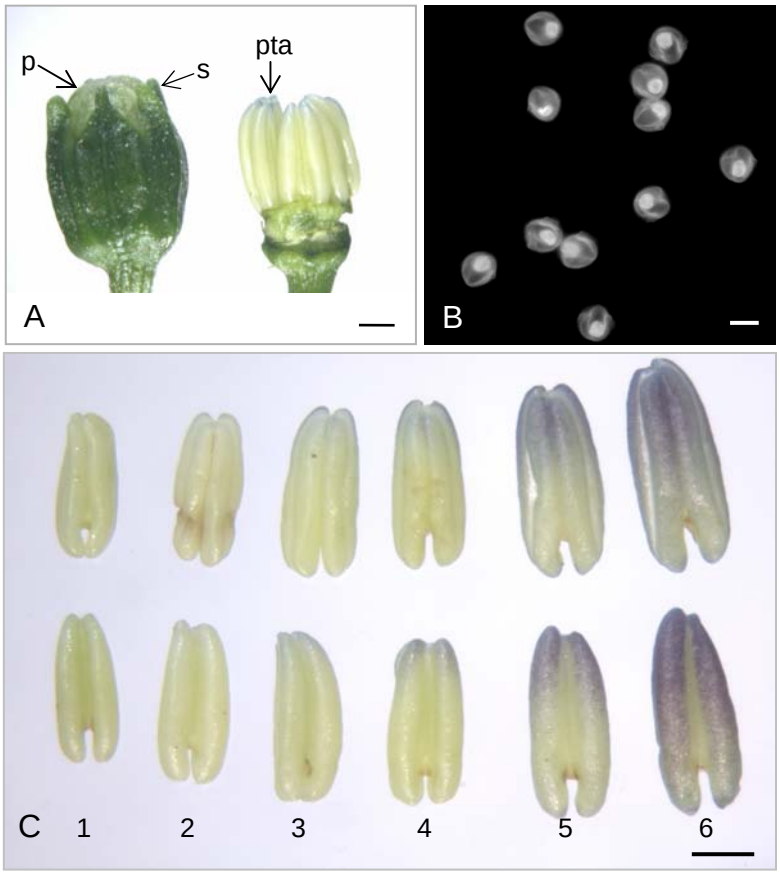

Figure 3, Chapter 3, page 47.

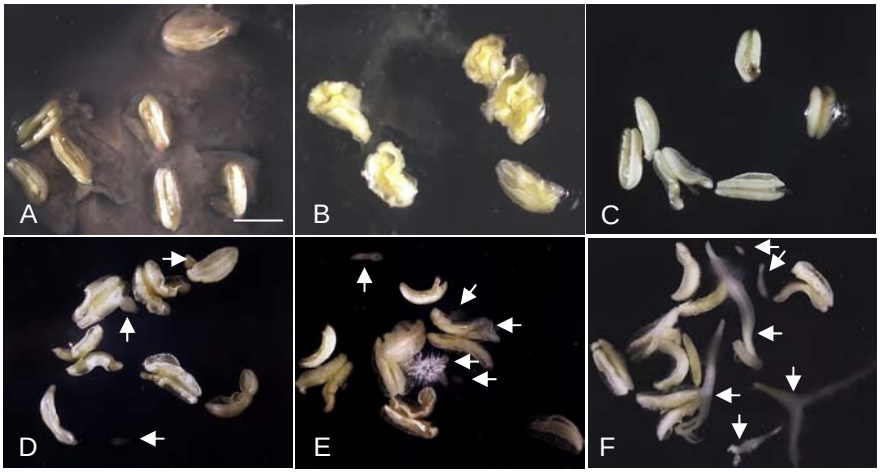

Figure 1, Chapter 4, page 64.

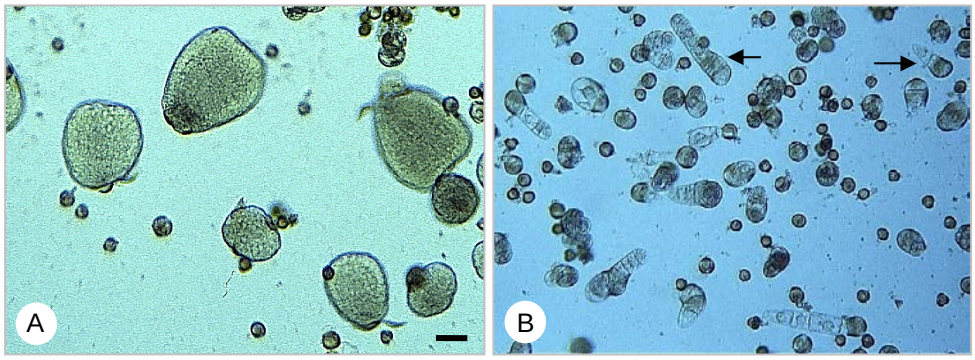

Figure 5, chapter 5, page 86.

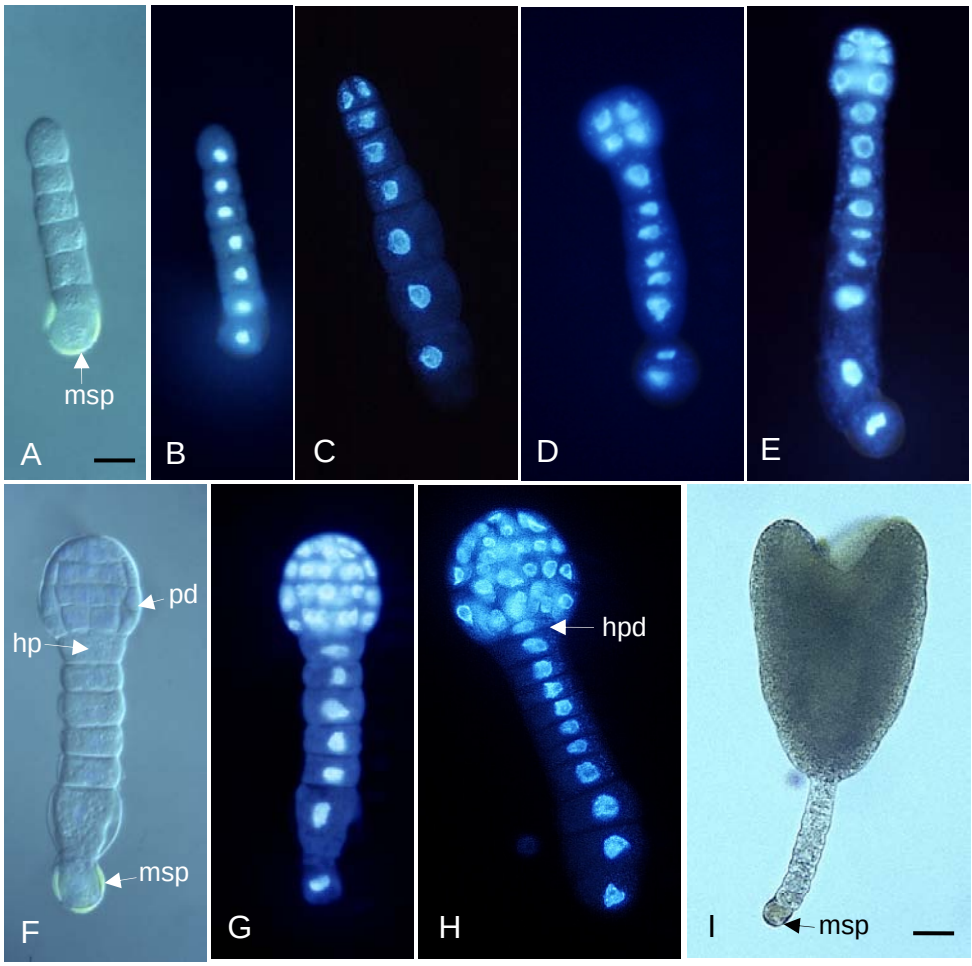

Figure 6, chapter 5, page 87.