Methodical improvements in microspore culture of *Brassica napus* L.

Methodische Verbesserungen in der Mikrosporenkultur

von *Brassica napus* L.

Dissertation

zur Erlangung des Doktorgrades

der Fakultät für Agrarwissenschaften

der Georg-August-Universität Göttingen

vorgelegt von

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geboren in Alfeld (Leine)

Göttingen, Dezember 2012

D7

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Tag der mündlichen Prüfung: 14.02.2013

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Chapter 1:

General Introduction

1.1 The agronomical importance of rapeseed

The amphidiploids species rapeseed (*Brassica napus* L.) belongs to the family of *Brassicaceae* which origins from a spontaneous hybridization (Song et al. 1988) between *B. rapa* (2n=20, AA) and *B. oleracea* (2n=18, CC). The combined rapeseed genome consists of 38 chromosomes. In contrast to spring forms that are mainly grown in Canada, northern Europe and China (Raymer 2002), winter forms require a vernalisation. The winter type *B. napus* is the main oilseed crop in Europe and parts of China. Historically, rapeseed oil is one of the oldest vegetable oils. In India, there are already indications for its use around 2000 BC. In the 16th and 17th century rapeseed became the most important oil crop in North-western Germany and the Netherlands. From the 17th century, cultivation took place on a larger scale. But its bitter taste due to high glucosinolate level made it useless for animal feed or human consumption. The improvement of rapeseed quality in the last four decades has led to the development of cultivars containing zero erucic acid in the oil and a low glucosinolate content in the meal, known as double zero cultivars, LEAR (for *low erucic acid rapeseed*) or Canola. Natural rapeseed oil contains about 50% erucic acid (Raymer 2002) whereas canola oil is limited by government regulation to a maximum of 2% erucic acid by weight in the USA (Food and Drug Administration, 2010). A further oilseed rape quality with modifications of the fatty acid composition is produced known as HOLLI for high oleic and low linolenic.

But intensive efforts of plant breeding companies were needed to produce high-yielding varieties (Becker 2011).

Since the improvement of quality, rapeseed is grown for production of animal feed, vegetable oil for human consumption and biodiesel. World production is growing rapidly with 50 million tons of rapeseed being produced in 2005/2006 and 59 million tons in 2010/2011 (FAOStat 2012). China, Canada, India, Germany and France are the five top producing countries of rapeseed. Germany produced 5.7 million tonnes in 2010/2011. For every ton of rapeseed about 400 kg of oil is being extracted.

1.2 The Doubled haploid technology: an important tool in plant breeding

1.2.1 An historical overview : The induction of haploids

In genetics and plant breeding the importance of haploid induction in diploid and polyploid species has been realised for a long time. In nature, haploids usually occur with an extremely low frequency of 0.001-0.01% (Bhojwani and Razdan 1996). Spontaneous induced haploids mostly occur through parthenogenesis, the embryo development from an unfertilized egg cell. Today, in vivo haploid production is used in *Solanaceae* species and *Zea* mays. In *Zea mays*, for example intraspecific pollination or pollen with specific genetic defects can stimulate the development of haploid plants (Bhojwani and Razdan 1996). The induction of haploids in vitro is influenced by two important processes: the stimulation of reduced female gametes (gynogenesis) or male gametes (androgenesis) to autonomously develop into haploid individuals (Bhojwani and Razdan 1996). In gynogenesis, the female egg-cell or synergid is stimulated to grow without being fertilized. This was first reported in barley by San Noeum (1976). Gynogenetic haploids developed by parthenogenesis (ovary/ovule culture) were observed mostly in sunflower (Yan et al. 1991) and sugar beet (Oelson and Bruun 1990). In rice, the gynogenetic haploids arise through synergid apogamy (Yang and Zhou et al. 1992). In androgenesis, the vegetative or generative nucleus of a pollen grain is stimulated to develop into haploids without fertilization. The occurrence of androgenic haploids in vivo has been reported for example in *H. vulgare* x *Hordeum bulbosum* (Bhojwani and Razdan 1996). Until 1964, the artificial production of haploids was attempted by the following methods: distant hybridization (selective elimination of chromosomes), delayed pollination, application of irradiated pollen, hormone and temperature treatments (Bhojwani and Razdan 1996). A major break-through in haploid breeding of higher plants was the work of the two Indian scientists Guha and Maheshwari in 1966 and 1967. They showed that isolated *in vitro* cultured anthers of *Datura innoxia* were able to form haploid embryos. In 1967, Bourgin and Nitsch obtained first haploid plants from isolated anthers of *Nicotiana*. The technique of haploid production through anther culture has been extended to numerous plant species including cereals, vegetable, oil, and tree crops. Androgenic plants arise through direct pollen embryogenesis *(Daucus carota*) or through pollen callusing (Hordeum vulgare). The basis of pollen and anther culture is that on an appropriate medium the microspores of some plant species can be induced to develop to sporophytic cells,

instead of pollen grains. A normal sexual gametophytic pattern of development changes into a sporophytic pattern of development. This change appears to be initiated in an early phase of the cell cycle. At this point, transcription of genes responsible for development is inhibited and genes responsible for sporophytic development are activated (Sunderland and Dunwell 1977). The consequence is that instead of pollen with the capacity to produce gametes and pollen tube, callus tissue or androgenic embryos form directly from the microspores. In previous studies, haploid production by culturing microspores within anthers was more efficient than by culturing isolated pollen (George et al. 1996). The role of anthers wall factors in pollen embryogenesis was suggested by a sample of historical studies of cultured anthers (Debergh and Nitsch 1973; Igusa and Raghaven 1978). Kott et al. (1988a) observed asynchronous pollen development in anther culture. By releasing toxic substances the older grains may suppress the androgenic response of younger grains. There are many problems associated with the anther culture; consequently, an isolated pollen culture offers some advantages: it is a haploid, single cell system which can circumvent these problems by elimination of the anthers. By Kameya and Hinata (1970) the first report was published, they observed callus formation in isolated pollen culture of an angiosperm (*Brassica oleracea* and the hybrid *B. oleracea* x *B. alboglabra*). The tedious process of dissection of individual anthers and the pre-culture of the anthers were avoided by the isolated pollen culture. The first protocol for haploid production from microspore culture from *Nicotiana* was published in 1969 by Nitsch.

1.2.2 Factors affecting the efficiency of microspore embryogenesis

In plant breeding programs the microspore culture technique plays an important role for efficient production of doubled haploids in rapeseed. Haploid embryos of *Brassica napus* from anther culture were first obtained in 1975 (Thomas and Wenzel). The protocol for isolated microspore culture followed in 1982 by Lichter. The efficiency of microspore culture is influenced by many different factors which seem to interact with each other. These factors include the growth conditions of donor plants (Ferrie and Keller 1995), microspore developmental stage (Kott and Beversdorf 1988a/b), induction of embryogenesis via temperature and/or chemicals (Shariatpanahi 2006, Zeng et al. 2010), and induction medium (Ilić -Grubor 1998, Ferrie and Keller 2007). Although since 1982 many scientists have studied these important factors to optimise and improve the efficiency of microspore culture, there

are still problems in doubled haploid breeding programs regarding the diploidisation frequency and direct plant conversion of microspore derived embryos.

1.2.2.1 Donor plant growth

Healthy, pest free donor plants are a prerequisite for successful and consistent microspore culture response (Ferrie and Caswell 2011). In contrast to field conditions, the cultivation of the donor plants in growth chambers allows the control of environmental growth factors. Temperature, humidity, photoperiod and light intensity are optimised for donor plant growth. The incidence of disease or insect infestation is minimised and can effectively be treated if necessary. A critical role plays the temperature at which donor plants are grown (Ferrie and Caswell 2011). Donor plants of *Brassica* species are usually grown at 20/15 °C and the bolting temperature is reduced to 10/5 °C. Ferrie and Keller (1995) reported that the cold temperature stress of the donor plants results in a higher frequency of microspore embryogenesis. The reduction of the temperature results in slower plant growth and buds at the appropriate developmental stage can be longer harvested. Generally, the buds developing first show a better response than those from the following flush (Dunwell et al. 1985). They reported that buds should be removed from the donor plant to prevent fruit formation and to maintain the plants young. Exposure of the donor plants to stress by treating the donor plants with substances which interfere with the normal development of pollen grains, such as gametocidal compounds like Feridazone-potassium (Bhojwani and Razdan 1996) could not promote androgenesis.

1.2.2.2 Microspore development stage

Selections of buds of the appropriate size with anthers containing immature pollen grains (microspores) are very critical for the induction of microspore embryogenesis. Correct bud size varies depending on the species. Generally, the pollen grains around the first pollen mitosis are most responsive (Bhojwani and Razdan 1996). Four *in vitro* androgenic pathways have been identified. The first pathway has been commonly observed in *Brassica napus* (Zaki and Dickinson 1995). By an equal division the microspore divides and the two identical daughter cells contribute to the sporophyte development. A distinct vegetative and generative cell is not formed. The second pathway is commonly observed in *Nicotiana tabacum* (Horner and Street 1978), *Hordeum vulgare* (Clapham 1971) and *Triticum aestivum*

(Ouyang et al. 1973). By a normal unequal division the uni-nucleate microspore divides and through further divisions in the vegetative cell the sporophyte arise. In the third pathway observed in *Hyoscyamos niger* the generative cell alone predominantly forms the pollen embryos. The vegetative cell does not divide or the division is limited or forms a suspensor-like structure (Raghaven 1978). In the fourth pathway reported in *Datura innoxia* (Sunderland et al. 1974), vegetative and generative cells are formed, but in contrast to the second pathway both divide further and participate in the development of the sporophyte. In contrast to other species, in cruciferous species the pollen mitosis II is completed in the pollen grain and does not occur in the pollen tube. In *Brassica* species, buds are harvested when the microspores are at the uni-nucleate to early bi-nucleate stage (Kott et al. 1988a). For determining the developmental stage of microspores usually the fluorescent stain DAPI (4´, 6-diamidino-2-phenylindole) is used. The microspore density should be between 50.000 and 100.000 cells and can be estimated using a haemocytometer. The size of the buds in *Brassica* species ranges from 2.0 mm up to 4.5 mm and varies with the genotype, the age of the donor plant as well as with the environmental conditions in which the plants are grown.

1.2.3 Induction of microspore embryogenesis

In *Brassica* microspore culture, a heat treatment is required to induce embryogenesis (Xu et al. 2007). The heat treatment of uni-nucleate microspores leads to a symmetrical instead of an asymmetrical division and to the development of an embryo instead of a mature pollen grain (Park et al. 1998). The gametophytic development is repressed and the sporophytic development is induced. Next to heat stress, the antimitotic agent Colchicine induces symmetric division during the pollen mitosis I (Park et al. 1998). Colchicine is known to enhance the embryogenesis (Zaki and Dickinson 1995). Cold treatment is less frequently used in *Brassica* species, heat stress and Colchicine could also be replaced by other stresses such as low levels of γ-irradiation, ethanol, modified atmosphere, and antimitotic agents (Xu et al. 2007). After addition of 0.1 µg/mL bleomycin, a glycopeptid antimitotic, Zeng at al. (2010) observed a two to fourfold increase in embryogenesis. Ferrie et al. (2005) found that two brassinosteroid compounds, 2,4-epibrassinolide and brassinolide, increased embryogenesis in different *Brassica napus* genotypes. The addition of the anti-auxin, p-chlorophenoxyisobutryc acid (PCIB) also increased the embryogenesis (Agarwal et al. 2006). Leroux et al. (2009) showed that the addition of ethylene synthesis inhibitors such as

aminoethoxyvinylglycine and cobalt chloride significantly increased embryo yields. They suggested that regulatory effects are exerted by the ethylene produced by the isolated microspores during the early processes of embryogenesis. Besides stress compounds, the induction media for cultivation of the isolated microspores also influence embryogenesis. In *B. napus*, a very efficient medium is NLN with 13% sugar and a pH of 5.8 (Lichter 1982). By using polyethylene glycol (PEG) to replace the sucrose in the culture medium, the embryogenesis could be enhanced (Ferrie and Keller 2007). In *Brassica*, Kott et al. (1988b) observed that microspore embryogenesis was restricted because of the production and/or the release of inhibitory substances during the first 24 hours of culture. The replacement of the media after 24 hours alleviates the toxicity and allows embryos to grow normally. Instead of changing the media, the addition of activated charcoal to trap toxic substances was also reported (Chatelet et al. 1999) but did not lead to increased embryogenesis.

1.2.4 Antimitotic induced diploidisation in microspore culture

For production of one-step homozygous, fertile doubled haploid lines, the antimitotic agent Colchicine is usually applied. The antimitotic agent can be applied to the single cell stage (microspore), to the *in vitro* regenerating embryos (Mohammadi et al. 2012) or plantlets and to haploid plants growing *in vivo* in the greenhouse. However, the *in vivo* treatment is very laborious and leads to insufficient diploidisation frequencies of 50% to 60%. Because of the toxicity of Colchicine, the plant development is significantly delayed. A few plant cells only are diploid after *in vivo* application. Consequently chimeric plants consisting of haploid and diploid cells are regenerated. The treated plants have to be pruned frequently resulting in the development of diploid shoots. This step is time consuming and poor seed yield requires additional greenhouse propagation of the material for performing field experiments. In comparison, treatment of isolated microspores with Colchicine is more efficient because completely diploid plants are regenerated. The chromosome doubling occurs at the single stage and prevents the regeneration of chimeric plants. Colchicine is used in lower concentrations and leads to higher diploidisation frequencies up to 94% (Möllers et al. 1994). A delayed plant development could not be observed as consequence of *in vitro* microspore diploidisation. Colchicine added directly to the microspore solution *in vitro* and subsequently incubated for 72 hours produced improved embryogenesis and showed no negative effects on embryo development (Iqbal et al. 1994). In comparison to other

antimitotic agents, Colchicine has to be applied in higher concentrations and because of its higher affinity for animal tubulin than for plant tubulin (Bartels and Hilton 1973). For this reason, this antimitotic agent is hazardous for human and environment. Although the concentration is considerably reduced in comparison to *in vivo* application, in microspore culture the Colchicine concentration can still be high with up to 1000 mg/L. Alternative antimitotic agents are needed having a higher affinity to plant tubulin and working in lower concentrations. In literature, the use of alternative antimitotic compounds such as Trifluralin, Oryzalin, Amiprophos-methyl and Pronamide were tested to affect chromosome doubling during the early stages of microspore culture and embryogenesis. The herbicides APM and Pronamide seem to have relevant properties. The mode of action of APM is similar to that of Colchicine. Colchicine binds to tubulin heterodimers during assembly of microtubules preventing their polymerisation (Margolis and Wilson 1977). In contrast to APM and Colchicine, Pronamide destabilizes microtubule by limiting the supply of proteins needed for microtubule development (Vaughn and Vaughn 1987). The herbicides Pronamide and APM had already been tested (Hanson et al. 1998, Hansen and Anderson 1996) but neither in combination with each other nor with Colchicine. Furthermore, dimethyl sulphoxide (DMSO) is often used to dissolve the antimitotic agent. Hitherto, DMSO has not been examined in addition with Colchicine or another antimitotic agent. The influence of DMSO in combination with Colchicine on embryogenesis, diploidisation frequency and direct plant conversion of microspore derived embryos has not been investigated.

1.2.5 Determination of ploidy level of microspore derived embryos

In haploid production, plants derived from microspore derived embryos can be haploid, diploid and polyploid. The non-haploids can originate from a) somatic tissue b) endoreduplication and fusion of nuclei or c) endomitosis during the early stages at microspore culture as well as from d) irregular gametes (unreduced gametes) formed by meiotic irregularities (Germanà 2011). However, in *Brassica napus*, evidence from short sequence repeat marker loci indicated that diploid and fertile plants (without an antimitotic treatment) are derived from spontaneous diploidisation of haploid gametes, rather than from unreduced gametes or somatic tissue (Takahira et al. 2011).

In *Brassica* species, the ploidy level of microspore derived plants could be determined at various stages. Generally, for practical breeding purposes, the ploidy level is determined at

the flowering stage by flower morphology, pollen fertility, flower size and seed set (Zhou et al. 2002). A variety of morphological methods are used to distinguish haploids from their diploid equivalents. The cell volume of plants is directly related to their ploidy level, for this reason, haploid plants are smaller in appearance because of their smaller cell size (Dunwell 2011). The precise ploidy level analysis is conducted via chromosome counts (Xu et al. 2007) or by using a flow cytometer. In plant breeding programs, flow cytometer measurements (FCM) analysis of ploidy level of microspore derived plantlets is usually made from young leaves of four weeks old plantlets cultivated in soil. For determination of diploidisation frequency after antimitotic treatment, the whole embryo could be chopped for the FCM. This method is less time consuming but embryos are completely destroyed. Another possibility is to cut the cotyledons of *in vitro* cultivated microspore derived embryos. After cutting the cotyledons the microspore derived embryos could further be cultivated *in vitro*.
In general, the flow cytometry belongs to the direct methods to determine the ploidy level of plants whereas indirect methods are based on guard cell and plastid sizes (Dunwell 2011). Additionally, mostly in anther or ovary culture it could be necessary to characterise the microspore derived regenerate at the genetic level to prove its origin by detection of homozygosity based on DNA markers. Because of the spontaneous chromosome doubling, diploid plants can be homozygous doubled haploids or heterozygous diploids produced by somatic tissue (Germanà 2011).

1.2.6 Direct conversion of microspore derived embryos to plantlets

An important step in the production of doubled haploid plants is the direct and rapid conversion of microspore derived embryos to plantlets. Fletcher et al. (1998) showed that the direct and quick plant regeneration ensures minimal occurrence of cytogenetic abnormalities. The development of microspores into an embryo can be direct or indirect. The direct and preferred path is similar to the zygotic embryo development. The embryos proceed through the globular, heart-shaped, torpedo, and cotyledonary stages (Ferrie and Caswell 2011). In most of the *Brassica* species the microspore derived embryos exhibit a poor direct conversion less than 30% (Bhojwani and Razdan 1996). On the shoot regeneration medium the hypocotyl elongates, the cotyledons turn green, a primary root develops but the plumule rarely develops a shoot. The observed indirect embryogenesis involves a number of irregular, asynchronous divisions resulting in callus which undergoes

organogenesis. The recalcitrant embryos regenerate plants through adventitious shoot bud differentiation or secondary embryogenesis from the epidermal cells of the hypocotyls (Chuong and Beversdorf 1985). Secondary embryogenesis and a callus phase require further sub-culturing which is very time consuming and cost intensive. By exposing microspore derived embryos to a period of low temperature (1-4 °C), Cegielska-Taras et al. (2002) observed an improvement in direct embryo to plant conversion of the microspore derived embryos. Zhang et al. (2006) showed an increase of direct embryo to plant conversion of microspore derived embryos after exposing embryos to a period of partial desiccation by air drying. Yingze et al. (1999) reported that the stage at which microspore derived embryos are transferred to solidified medium is critical for the direct embryo to plant conversion of microspore derived embryos. Some reports (Tian et al. 2004, Zhang et al. 2006) showed the significant influence of the culture media on direct shoot development. Developmental stage of the microspore derived embryos, culture medium, and culture conditions are critical factors and have to be optimised in order to enhance direct plant conversion of microspore derived embryos.

1.2.7 The importance of the genotype

The efficiency of microspore culture varies among genotypes within a species. Baillie et al. (1992) reported that one of the most important factors influencing microspore culture response was the genotype of the plant material. In *Brassica napus*, the Canadian summer rapeseed Topas DH4097 is highly embryogenic (10%) and is usually used for many basic research studies (Ferrie and Keller 1995, Ferrie and Caswell 2011). The genotype dependence is often observed in tissue culture techniques (Ferrie and Caswell 2011). In 1983, Kasha and Sequin-Swartz reported that haploidy in nature is controlled by one single gene in *Solanaceae*, known as hap gene (haploid inducer gene). They suggested that *in vitro* androgenesis is under genetic control, and this trait could be transferred from responsive to original non responsive clones in *Solanaceae*. The process of plant development is regulated by many genes. For example, in *Arabidopsis* the WUSCHEL (WUS) gene is required for stem cell identity, whereas the *CLAVATA1*, *2*, and *3* (*CLV*) genes promote organ initiation (Schoof et al. 2000). Endrizzi et al. (1996) analysed the function of another gene known as *SHOOT MERISTEMLESS* (STM) gene which is required for maintenance of undifferentiated cells in shoot and floral meristems and acts on a different regulatory level than the WUS gene.

Studying the molecular mechanism by gene characterization via microarray or the production of mutants is necessary to achieve progresses in plant breeding. In 2002, Boutellier et al. identified a gene known as BABY BOOM (BBM) which is up-regulated during microspore embryogenesis in *Brassica* and belongs to the AP2-ERF (APETALA2 ethylene-responsive factor) transcription factor family. In *Brassica napus* two BBM genes were found with 98% similarity to each other. Next to BBM gene, Malik et al. (2007) identified a number of genes which were up-regulated in embryogenic microspores. Their characterisation could serve as a basic for further genetic researches (Ferrie and Möllers 2011). Those genes which are involved in embryogenic pathway used as candidate genes during microspore embryogenesis could be helpful to select superior genotypes for DH breeding programs. In general, little is known about the genetic mechanism which controls the limited steps regarding embryogenic potential, diploidisation frequency and direct plant conversion of microspore derived embryos in the production of doubled haploid lines.

1.2.8 Advantages of doubled haploid technology in rapeseed

In plant breeding the most important application of the microspore culture technique is the production of stable, homozygous doubled haploids (DH) in a single generation. DH plants have been successfully applied in breeding programs when completely homozygous lines are quickly needed (Palmer et al. 1996). For example, in hybrid breeding programs homozygous lines which are produced by Doubled haploid (DH) technology, are a prerequisite for the production of hybrids. The isolated microspore technology has the potential to produce large numbers of DH plants (Palmer et al. 1996). Their genetic segregation is simplified because in homozygous individuals, recessive genes are not masked by dominant ones (Friedt and Zarhloul 2005). Furthermore, in general, phenotypic differentiation of lines is easier because of their homozygosity (Becker 2011). In contrast to DH technology method, in conventional breeding, several segregating generations must be grown in order to reach a certain level of homozygosity (Soriano 2008). For this reason, applying the DH technology the release a new variety is possible after four to five years (Friedt and Zarhloul 2005). Shortening the breeding cycle by up to two years is one of the advantages (Becker 2011).

The objectives of this study

were to:

- investigate the diploidisation of the haploid genome at the single-cell microspore stage with alternative antimitotic agents compared to Colchicine
- analyse alternative antimitotic agents in combination with or without Colchicine and the influence of DMSO on diploidisation frequency and direct embryo to plant conversion of microspore derived embryos
- enhance the antimitotic induced diploidisation frequency of microspore derived embryos
- improve direct embryo to plant conversion of microspore derived embryos
- analyse winter oilseed rape cultivars and their F1-crosses regarding their diploidisation frequency and direct embryo to plant conversion of microspore derived embryos

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Chapter 2:

Ploidy level and direct embryo to plant conversion following APM, Pronamide, Colchicine and DMSO treatment of microspore derived embryos of winter oilseed rape cultivars and their F1-crosses (*Brassica napus* L.)

2.1 Abstract

In oilseed rape, the major drawbacks in the efficient application of doubled haploid technology are an insufficient Colchicine induced diploidisation frequency of about 60% and a low direct embryo to plant conversion.

The main objective of this study was to enhance the antimitotic induced diploidisation frequency without a negative influence of the antimitotic agent on the ability of microspore derived embryos to convert directly to plantlets (direct embryo to plant conversion rate). For that reason, the following four experiments were conducted to study the effect of less toxic alternative antimitotic agents with a high affinity to plant tubulin, the effect of different antimitotic agents in combination with each other, the effect of different Colchicine concentrations and the effect of DMSO, often used as solvent for several antimitotic agents on diploidisation and direct embryo to plant conversion frequencies of microspore derived embryos. Furthermore, the spontaneous and Colchicine induced diploidisation frequency of 13 winter oilseed genotypes including cultivars and F1-hybrids and the direct embryo to plant conversion rate were analysed. The antimitotic agents were applied for 72 hours directly to freshly isolated microspore suspensions. The developed microspore derived embryos were cultivated at cotyledonary stage on solidified culture media. After four-week cultivation the ploidy level and the direct embryo to plant conversion rate were determined.

In the first experiment eight genotypes were tested showing a diploidisation frequency varying from 40% to 64%. Regarding all antimitotic treatments, the diploidisation ranged from 33% (3 µM APM, 72 hours) to 70% (25 µM Colchicine, 72 hours). When combining the antimitotic agents no synergistic effect on the diploidisation frequency could be detected. The antimitotic agents had no significant effect on direct embryo to plant conversion rate.

In the second experiment four genotypes were tested showing a Colchicine induced diploidisation frequency ranging from 58% to 66%. The different Colchicine treatments (250,

150, 125, 25 µM) resulted in frequencies varying from 59% (150 µM, 72 hours) to 77% (250 µM, 48 hours). The Colchicine treatment had no significant effect on direct embryo to plant conversion rate.

In the third experiment, for the four tested genotypes no significant effect of DMSO on diploidisation and direct embryo to plant conversion frequency could be observed.

In the fourth experiment, 13 genotypes (cultivars and F1-hybrids) were analysed. The spontaneous induced diploidisation frequency showed a wide range from 15% to 69% with a mean of 34%. The Colchicine induced frequency varied from 42% to 83% with a mean of 68%. For all tested genotypes, the direct embryo to plant conversion rate ranged from 2% to 35%.

Regarding all experiments the induced diploidisation and direct embryo to plant conversion rate of microspore derived embryos was genotype dependent. Further investigations are necessary to gain an efficient diploidisation frequency of about 90%.

2.2 Introduction

Although, microspore culture of *Brassica napus* is already established in plant breeding processes and regarded as routine technique for production of doubled haploid plants, there are still some bottlenecks which limit the efficiency of this technique. One of the problems is the ability of chromosome doubling. Oilseed rape shows a low spontaneous induced diploidisation rate of about 30% (Möllers and Iqbal 2009). The spontaneous and antimitotic induced diploidisation rate is genotype dependent. At present, Colchicine is the most common antimitotic agent which can be applied to isolated microspores; microspore derived embryos or regenerated plants (Xu et al. 2007). Recent studies examined the optimised method to apply Colchicine, including concentrations from 1 µM to 1000µM and times of duration from six hours to three days. Different studies (Zhou et al. 2002, Weber et al. 2005, Möllers 2006) reported that the diploidisation frequency has a wide range from 10% to 95%. Results from a survey showed that the microspore diploidisation in winter rapeseed is applied by comparatively few plant breeding companies and that the mean of diploidisation efficiency of about 60% is still insufficient (Möllers 2006). However, high reproducible diploidisation frequencies of about 90% are sufficient. The highest diploidisation rates of 90% to 94% (Möllers et al. 1994) were determined after treatment with 125 µM Colchicine

for 24 hours and 250 µM Colchicine for 72 hours in microspore culture. Colchicine has to be used in comparatively high concentrations because of its low affinity to plant tubulin. These high concentrations are more problematic, since Ravelli et al. (2004) reported that Colchicine could also depolymerise animal and human microtubules. Besides the risk of the hazardous exposure to humans and environments it also requires waste storage and an expensive disposal. Due to that risk potential, alternative mitotic agents are needed, having a higher affinity for plant tubulin and a preferably lesser affinity for animal and human tubulin. Further researches evaluated the effects of the less toxic antimitotic agents such as Amiprophos-methyl and Pronamide on embryogenesis and chromosome doubling during microspore culture (Hansen and Anderson 1996, Hansen et al. 1998, Klìma et al. 2008.) In contrast to Colchicine, the phosphoric amide APM has been reported to show higher affinity to plant than for human tubulin (Bartels and Hilton 1973, Morejohn and Fosket 1984, Morejohn et al. 1987). In Literature, Hansen and Anderson (1996) stated that APM has *in vitro* effects on plant microtubules similar to those of Colchicine, however, with a much higher binding affinity (Sree Ramulu et al. 1991). Hansen and Anderson (1996) reported a stimulating effect of APM on embryo formation at low concentrations. In contrast to Colchicine which binds to tubulin heterodimers during assembly of microtubules preventing their polymerisation (Margolis and Wilson 1977), Pronamide destabilises the microtubule or limits the supply of proteins needed for microtubule development (Vaughn and Vaughn 1987). Furthermore, in contrast to its high affinity for plant tubulin, Pronamide shows no affinity for animal or human tubulin.

The main objective of this work was to study possible synergistic effects of simultaneous application of APM, Pronamide and Colchicine on the microspore induced diploidisation rate with the aim to possibly replace the more toxic Colchicine. A further objective was to study the genetic variability for spontaneous and induced diploidisation rate and for the direct embryo to plant conversion.

2.3 Materials & Methods

2.3.1 Donor plant material

Microspore donor plants of winter oilseed rape cultivars Adriana, Charly, DSV1, DSV2, Express 617, Krypton, Komando, Favorite and Oase and F1-donor plants of the crosses Komando × Express 617, Express 617 × Charly, Charly × Krypton, Krypton ×DSV1, DSV1 × Adriana, Adriana × Oase, Oase × NK Beauty and Favorite x DSV2 were used for the experiments. F1-donor plants were clonally propagated *in vitro* and plantlets were transferred to soil whenever donor plant material was needed.

2.3.2 Donor plant growth

Seeds of winter oilseed rape line cultivars and breeding lines Charly, DSV1, DSV2, Favorite and Oase (DSV, Lippstadt, Germany); Komando and Krypton (KWS Saat AG, Einbeck, Germany), NK Beauty (Syngenta Seeds, Bad Salzuflen, Germany), Adriana (Limagrain, Peine, Germany) and Express 617 (NPZ Lemke, Hohenlieth/Malchow, Germany) were provided by the breeding companies. The seed-derived and *in vitro* propagated plantlets were grown in the glasshouse until they reached the four- to six-leaf-stage. They were then transferred for vernalisation to a controlled climate chamber at 4 °C with a short photoperiod (eight hours/day) for a period of eight weeks. After vernalisation, the plants were re-potted to 13 cm plant pots filled with compost soil and grown in the greenhouse. On appearance of the first flower buds, the plants were transferred to an environmentally controlled growth chamber with a 16 hours photoperiod, a day/night temperature of 10/5 °C, light intensity of 400 µmol m^{-2} s^{-1} (Li-1400 Data Logger, Licor Biosciences, Nebraska, Canada) and a relative humidity of 65%. Before transferring, the insecticide Confidor WG® 70 was applied prophylactically against aphids. The plants were fertilised once a week with N:P:K (15:11:15) and watered three times a week.

2.3.3 Bud selection and microspore culture

Dependent on genotype flower buds with a length of 2.5 mm to 4.5 mm were selected from the primary and lateral inflorescences and collected in flasks. To avoid microspore degeneration the harvested buds were kept on ice. Flower buds were surface-sterilised for 5

minutes with periodic agitation in a 1% calcium hypochlorite solution with a few drops Tween 20 and were then rinsed thrice with sterile deionised water. For microspore isolation 16 buds were transferred to a nylon sieve (Wilson Sieves, Nottingham, UK, 5 cm diameter, mesh pore size 40-50 µm) with 7 mL NLN-media (Lichter, 1982; Duchefa, Harrlem, The Netherlands). By gently crushing the buds with the end of a pestle the microspores were released. The pestle and the sieve were rinsed with 5 mL NLN media to collect microspores in the Petri dish. The microspore suspension was transferred to a 12 mL glass tube, which was then centrifuged at 100 x g for 5 minutes. The supernatant was decanted and NLN media was added to resuspend the microspore pellet of microspore. After a second centrifugation at 100 x g for 5 minutes, the supernatant was discarded again and the microspore pellet was resuspended in NLN medium and transferred to a new plastic Petri dish (92 x 16 mm, Sarstedt AG & Co., Nürnbrecht, Germany). Depending on the experiment antimitotic agents in different concentrations were added to the NLN medium so that the microspores were suspended in the Petri dish in a total volume of 12 mL medium. The concentration in the final suspension was determined to range between 50.000 and 80.000 microspores per millilitre culture medium. The Petri dish was sealed with two layers of Parafilm and was incubated in darkness at 32 °C for 48 to 72 hours. After the heat treatment the microspore suspension was transferred again to a tube and was centrifuged (100 x g, 5 min). The supernatant was removed and the pellet was resuspended with 12 mL NLN-media in a new Petri dish which was incubated for eight days at 28 °C in darkness. Then the Petri dish was transferred to a shaker (80 rpm) under 12 hours light (Osram Cool white) at 25 °C. Depending on the experiment, the number of buds used per preparation was increased two to three fold. For most of the genotypes the first yellowish microspore derived embryos became visible after 10 to 14 days in culture. After two weeks in culture microspore derived embryos were diluted by transferring them with a forceps to new Petri dishes (20 microspore derived embryos with 25 mL of NLN media per Petri dish).

2.3.4 Antimitotic treatment of microspore culture

Stock solutions of Colchicine (MW: 399.5, 0.005 M; Serva GmbH, Heidelberg, Germany), Pronamide (MW: 256.1, 0.01 M; Propyzamid, Serva GmbH, Heidelberg, Germany) and Amiprophos-methyl (MW: 304.3, 0.01 M; Serva GmbH, Heidelberg, Germany) were prepared. APM and Pronamide were dissolved in dimethyl sulphoxide (DMSO, Serva GmbH,

Heidelberg, Germany). Colchicine was dissolved in sterilised water and the solution filter-sterilised using a bacterial filter (Sartorius NML, pore size 0.2 µm). Appropriate volumes from the stock solution were added to freshly isolated microspore cultures to give the desired final antimitotica concentration. For experiments with DMSO, appropriate volumes from the Colchicine stock solution and DMSO were applied to freshly isolated microspore cultures to give the desired final antimitotic concentration.

2.3.5 Regeneration of microspore derived embryos to plants and scoring of direct embryo to plant conversion frequency

After one to two weeks of microspore derived embryo growth at reduced densities, the microspore derived embryos at the late cotyledonary stage (0.5-0.7 cm) were transferred to agar solidified media in plastic boxes (10.5 x 8 x 5 cm, Volume 0.24 L, Huhtamaki GmbH & Co.KG, Alf, Germany) with agar (1%, Plant Agar, C.E. Roeper GmbH, Germany) solidified Gamborg B5 with 0.1 mg/L gibberellic acid (Gamborg B5 and GA_3, Duchefa, Haarlem, The Netherlands). The plastic boxes each containing eight microspore derived embryos were incubated in a culture room at 25 °C and a 12-hour photoperiod (Osram Cool white). After a period of four weeks the rate of direct embryo to plant conversion and the ploidy level was determined.

2.3.6 Ploidy level determination by flow cytometry

After determination of direct embryo to plant conversion, regenerated plantlets were used for ploidy determination. For this a small piece of leaf, shoot (directly regenerated plantlets) or embryo tissue was placed in a Petri dish with 0.5 mL of water and was chopped into small pieces with a razor blade. After adding 1 mL of the fluorochrome solution 4´6-diamidino-2-phenylindole solution (DAPI, Partec GmbH, Münster) to the chopped material, the suspension of cellular debris was filtered through nylon gauze (40 µm). Immediately, the filtrate was analysed with the Partec Cell Analyser CA-II flow cytometer (Partec GmbH, Münster). Leaf tissue from *in vitro* cultured haploid and diploid *Brassica napus* was used as standard. By comparing the DNA peaks with the identified haploid and diploid standard, the ploidy level of the samples was assigned: haploid, diploid, tetraploid or mixploid.

2.3.7 Experimental design and statistical analysis

Depending on the experiment between four and 17 genotypes were used in combination with two to eight different antimitotic treatments. Per genotype and antimitotic treatment, three independent experiments were performed, and in each experiment ploidy level and percentage of direct embryo to plant conversion was determined using 48 microspore derived embryos (six plastic boxes with eight embryos). A two-way analysis of variance (ANOVA) was performed as series of experiments (E=3) with the factors 'genotype' (G) and 'antimitotic treatment' (T) by using PLABSTAT software (Utz 2011) considering the genotypes and experiments as random. ArcSin-transformed data were used in the ANOVA and significant differences between genotypes, treatments and interactions was assessed by LSD calculated at α=0.05 confidence level.

2.4 Results

2.4.1 Effect of an APM, Pronamide and Colchicine treatment of microspores on ploidy level and direct embryo to plant conversion of microspore derived embryos

This experiment was performed to investigate the effect of APM and Pronamide alone, in combination together, and with and without Colchicine on diploidisation of microspores and on the direct embryo to plant conversion rate of microspore derived embryos. Furthermore, the effect of a permanent Colchicine treatment at a low concentration (25 µM) without subsequent washing of the microspores was tested in comparison to a standard treatment (250 µM Colchicine for 72 hours). The different treatments are shown in Tab. 1. The antimitotic agent treatments were applied to isolated microspores of F1-plants derived from eight oilseed rape genotypes.

The analysis of variance showed highly significant effects of the genotypes and the antimitotic treatments on the ploidy level of the microspore derived embryos and plantlets (Tab. 2). Variance components showed a predominant effect of the genotype on the frequency of diploid embryos, whereas a predominant effect of the antimitotic treatment on the occurrence of haploid and tetraploid embryos was observed. The interaction effects genotype x treatment were also significant. Heritabilities for the ploidy levels ranged from

0.53 for haploid embryos to 0.82 for diploid embryos. The diploidisation frequency as a mean over the eight genotypes and three experiments ranged from 33.4% for the treatment 3 µM APM for 72 hours to 69.6% for the 25 µM Colchicine 72 hours treatment (Tab. 1). The untreated control showed a mean diploidisation frequency of 36.4%. The permanent treatment with low concentrations of Colchicine (25 µM) led to the highest number of tetraploid and to the second lowest number of haploid embryos. Among the oilseed rape genotypes the diploidisation rate without antimitotic treatment ranged from 20.6% for DSV1 x Adriana to 69.3% for Favorite × DSV2 (Tab. 3). With the most effective treatment, 25 µM Colchicine for 72 hours, the diploidisation frequency ranged from 25.6% for Adriana × Oase to 87.6% for Charly × Krypton.

The variance components also showed a highly significant effect of the genotypes on the trait direct embryo to plant conversion (Tab 2). There was no significant effect of the antimitotic treatments on the direct embryo to plant conversion. The mean direct embryo to plant conversion rate over all treatments was 17.5%. For the eight oilseed rape genotypes the mean direct embryo to plant conversion rate of the microspore derived embryos ranged from 5.3% for Favorite × DSV2 to 28.3% for DSV1 × Adriana (data not shown). By APM (1.5µM), Pronamide (1.5µM) and Colchicine (25µM) treatment (3 µM) the highest mean of 23.1% was achieved varying from 3.0% for Komando × Express to 38% for DSV1 × Adriana. By treatment with 25 µM Colchicine for 72 hours the direct embryo to plant conversion rate ranged from 2.1% for Favorite × DSV2 to 31.6% for Krypton × DSV2 (data not shown). The genotypes showed a high heritability of 0.92 for the trait direct plant conversion of microspore derived embryos.

2.4.2 Effect of an APM, Pronamide and Colchicine treatment of microspores on embryogenesis

The microspore culture response varied among the tested genotypes. The genotypes Komando x Express, and Charly × Krypton were highly embryogenic. Between 500 and 1000 embryos per Petri dish and experiment were counted. The genotypes Adriana × Oase, DSV1 × Adriana, and Adriana were poorly embryogenic. Between 20 and 50 embryos per Petri dish and experiment were counted. Compared to no antimitotic treatment, all genotypes showed the highest embryogenesis after previous treatment of 250 µM Colchicine, followed by 25 µM each incubated for 72 hours. The 25 µM treatment with a permanent incubation

achieved a reduced embryogenesis. Several abnormal developed embryos were observed. All genotypes showed a low embryogenesis by treatment with 3 µM APM. The embryogenesis by treatment with Pronamide was comparable to the control (no antimitotic treatment).

Tab. 1 Ploidy level [%] and direct plant conversion rate [%] of microspore derived embryos after their antimitotic treatments. Depicted are means over eight genotypes and three experiments.

		Ploidy level [%]			Direct embryo to plant conversion [%]
Antimitotic treatment	**Duration time (h)**	**1n**	**2n**	**4n**	
Control (no antimitotic treatment)	-	58.1	36.4	5.6	14.2
25 µM Colchicine	Permanent	12.6	59.5	27.9	13.9
25 µM Colchicine	72	8.4	69.6	22.1	15.2
250 µM Colchicine	72	14.6	67.2	18.2	16.1
1.5 µM APM +1.5 µM Pronamide	72	46.4	48.6	5.0	16.9
1.5 µM APM +1.5 µM Pronamide +25 µM Colchicine	72	29.7	61.1	9.2	23.1
3 µM APM	72	63.5	33.4	3.2	18.1
3 µM Pronamide	72	44.1	52.0	3.9	22.2
Mean T		34.7	53.5	11.9	17.5
LSD5%		16.5	15.3	11.3	8.7

Tab. 2 Degrees of freedom (DF), mean squares, variance components and F-values for ploidy level [%] and for direct plant conversion rate [%] of microspore derived embryos after antimitotic treatment.

Source	**DF**	**Mean squares**				**Variance components**				**F-value**			
		Ploidy level			**Direct embryo to plant conversion**	**Ploidy level**			**Direct embryo to plant conversion**	**Ploidy level**			**Direct embryo to plant conversion**
		1n	**2n**	**4n**		**1n**	**2n**	**4n**		**1n**	**2n**	**4n**	
Genotype (G)	7	328	507	251	282	7.2	17.3	6.2	43.3	2.1*	5.5**	2.4**	12.6**
Treatment (T)	7	5927	1682	1879	1890	227.2	57.9	66.6	1.4	12.5**	5.8**	6.7**	1.3
Experiment (E)	2	20	46	43	53	-1.5	-0.7	-0.9	-0.1	0.2	0.5	0.4	0.9
GT	49	474	291	281	275	106.5	66.5	59.5	16.6	3.1*	3.2**	2.7**	1.6*
GE	14	122	92	102	85	-4.1	0.0	-0.1	-0.6	0.8	1.0	1.0	1.0
TE	14	75	96	162	58	-10.0	0.5	7.4	-3.9	0.5	1.1	1.6+	0.7
Heritability										**0.53**	**0.82**	**0.59**	**0.92**

+, *, ** denotes significant at P=10, 5 and 1%

Tab. 3 Doubled haploid plantlets [%] of microspore derived embryos per genotype after antimitotic treatment. Depicted are means over three experiments.

Genotype	Mean	Control	Permanent incubation	Duration time of 72h					
		Antimitotic treatment **Doubled haploid plantlets [%]**							
		No antimitotic treatment	**25 µM Colchicine**	**25 µM Colchicine**	**250 µM Colchicine**	**1.5 µM APM +1.5 µM Pronamide**	**1.5 µM APM +1.5 µM Pronamide +25 µM Colchicine**	**3 µM Pronamide**	**3 µM APM**
Favorite × DSV2	63.9	69.3	59.2	77.9	82.1	71.7	65.2	41.4	44.3
Charly × Krypton	58.2	23.8	58.2	87.6	81.3	46.4	55.3	82.5	30.7
Express × Charly	56.1	32.9	80.4	64.6	61.6	83.2	44.0	35.8	46.5
Komando × Express	54.9	45.8	65.8	73.3	74.4	35.0	62.8	42.1	39.7
Oase × NK Beauty	54.0	36.5	51.3	87.5	60.8	34.3	91.3	42.8	27.2
Krypton × DSV2	53.0	29.2	57.1	81.1	45.2	45.6	68.8	74.7	22.6
DSV1 × Adriana	47.2	20.6	44.7	59.0	70.8	60.8	47.2	46.2	28.1
Adriana × Oase	40.4	32.8	59.6	25.6	60.9	12.0	54.0	50.6	27.6
Mean G	53.5	36.4	59.5	69.6	67.2	48.6	61.1	51.0	33.4
LSD5% G	8.9								
LSD5% T						15.3			
LSD 5% G x T	8.4								

2.4.3 Detailed analysis of Colchicine concentration and treatment duration on ploidy level and direct embryo to plant conversion of microspore derived embryos

In the previous experiment Colchicine treatment of isolated microspores was identified as most effective to induce diploidisation. The following experiment was performed to study more in detail the effect of different Colchicine concentrations (25 to 250 µM) and treatment durations (48 to 72 hours; see Tab. 4) on ploidy level and direct embryo to plant conversion of microspore derived embryos.

There was a highly significant effect of the different treatments on the percentage of haploid, diploid and tetraploid microspore derived embryos but only a weakly significant effect on the direct embryo to plant conversion (Tab. 5). Differences between the genotypes were only significant for the percentage of haploid and tetraploid microspore derived embryos. In the control variant (no antimitotic treatment) the mean diploidisation rate was 32.7% whereas in the most effective Colchicine treatment with 250 µM for 48 hours a diploidisation rate of 77.3% was achieved (Tab.4). The mean direct embryo to plant conversion rate ranged from 6.5% for the 125 µM and 72-hour treatment to 22.7% for the 250 µM and 72-hour treatment. Genotype and treatment interaction was significant for the different ploidy levels and for the direct embryo to plant conversion. Heritabilities were high for the percentage of haploid and tetraploid embryos as well as for the direct embryo to plant conversion rate and low for the percentage of diploid embryos. In the control treatment the spontaneous diploidisation rate ranged from 23.8% for Charly × Krypton to 64.1% for Krypton × DSV2 (Tab. 6).

Tab. 4 Ploidy level [%] and direct plant conversion rate [%] of microspore derived embryos after their Colchicine treatments. Depicted means over four genotypes and three experiments.

	Ploidy level [%]			Direct embryo to plant conversion [%]
Colchicine treatment	**1x**	**2x**	**4x**	
250 µM, 72 hours	14.3	68.2	17.5	22.7
250 µM, 48 hours	6.9	77.3	15.8	8.5
150 µM, 72 hours	6.3	58.9	34.8	11.2
125 µM, 72 hours	1.1	61.2	37.7	6.5
25 µM, 72 hours	3.6	70.6	23.7	11.8
0 µM	62.9	32.7	4.4	14.2
Mean T	15.9	61.5	22.7	12.5
LSD5% T	13.6	15.8	14.5	10.1

Tab. 5 Degrees of Freedom (DF), mean squares, variance components and F-values for ploidy level [%] and for direct embryo to plant conversion rate [%] of microspore derived embryos after Colchicine treatment

Source	**DF**	**Mean squares**				**Variance components**				**F-value**			
		Ploidy level			**Direct embryo to plant conversion**	**Ploidy level**			**Direct embryo to plant conversion**	**Ploidy level**			**Direct embryo to plant conversion**
		1x	**2x**	**4x**		**1x**	**2x**	**4x**		**1x**	**2x**	**4x**	
Genotype (G)	3	713	79	776	448	37.5	0.6	39.7	22.3	19.2^{**}	1.2	12.9^{**}	9.6^{**}
Treatment (T)	5	3751	1101	1404	274	298.8	80.8	102.8	13.9	22.6^{**}	8.5^{**}	8.2^{**}	2.5^{+}
Experiment (E)	2	7	171	206	36	-4.6	5.0	1.7	-0.8	0.1	3.3	1.3	0.6
GT	15	166	130	171	108	43.0	20.4	17.4	20.4	4.5^{**}	1.9^{+}	2.8^{**}	2.3^{*}
GE	6	117	51	165	65	13.4	-2.9	-0.8	0.8	3.2^{*}	0.8	2.7^{*}	1.1
TE	10	61	104	57	42	6.0	8.7	60.5	-1.1	1.7	1.5	0.9	0.9
Heritibility		**0.95**	**0.13**	**0.92**	**0.90**								

$^{+}$, *, ** denotes significant at P=10, 5 and 1%

Tab. 6 Doubled haploid plantlets [%] of microspores derived embryos per genotype after Colchicine treatment. Depicted are means over three experiments.

Genotype	Mean	Colchicine treatment Doubled haploid plantlets [%]					
		250 µM, 2d	250 µM	150 µM	125 µM	25 µM	Control 0 µM
		Duration time 48h	Duration time 72h				
Komando x Express	65.9	73.5	78.5	62.5	56.2	73.1	45.5
Charly x Krypton	59.1	75.5	81.7	40.3	66.5	66.5	23.8
Krypton x DSV2	64.1	83.6	51.7	64.6	78.1	28.6	64.1
Express x Charly	57.9	76.6	60.9	54.8	57.6	64.6	33.0
Mean G	61.5	77.3	68.2	58.9	61.2	70.6	32.7
LSD5% G	4.1						
LSD5% T	15.9			15.9			
LSD5% G x T	9.0						

2.4.4 Effect of a Dimethyl sulphoxide (DMSO) plus Colchicine treatment on ploidy level and direct embryo to plant conversion of microspore derived embryos

The previous experiments showed that a treatment with antimitotic agents in different concentrations and treatment durations led to a mean diploidisation rate of not more than 77.3% (Tab. 6) The following experiment was performed to detect a potential influence of DMSO (3%, 0.3%, 0%) in combination with Colchicine (250 µM for 72 hours) on diploidisation frequency and on direct embryo to plant conversion of microspore derived embryos.
However, in the analysis of variance no significant effect of the treatment on diploidisation frequency and direct embryo to plant conversion of microspore derived embryos could be detected (Tab. 7). As in previous experiments a significant effect of the genotypes on the direct embryo to plant conversion rate of microspore derived embryos was found. Colchicine with 0.3% DMSO led to the highest mean of diploids with 70.0%, followed by 65.0% diploids in the control variant (no antimitotic treatment and DMSO) and 54.6% diploids in the 3% DMSO treatment (data not shown). Surprisingly, 22.1% tetraploids were found in the control variant (0% DMSO) which is in contrast to only 6.5% in both DMSO treatments. No decreased embryogenesis was observed after previous application of DMSO (0.3% and 3%) plus Colchicine (data not shown).

Tab. 7 Degrees of Freedom (DF), mean squares, variance components and F-values for ploidy level [%] and for direct plant conversion rate [%] of microspore derived embryos after DMSO treatment

Source	DF	Mean squares				Variance components				F-value			
		Ploidy level			Direct embryo to plant conversion	Ploidy level			Direct embryo to plant conversion	Ploidy level			Direct embryo to plant conversion
		1x	2x	4x		1x	2x	4x		1x	2x	4x	
Genotype (G)	3	999	686	161	241	104.0	71.4	11.5	20.6	15.9^{**}	16.0^{**}	3.2^{+}	4.4^{*}
Treatment (T)	2	1254	306	834	471	0.1	-14.9	33.5	26.0	1.0	0.6	1.9	3.0
Experiment (E)	2	37	129	67	10	-1.1	7.0	4.1	-5.4	0.7	2.9	3.8^{+}	0.1
G x T	6	1253	484	432	159	396.8	147.4	127.4	34.5	20.0^{**}	11.3^{**}	8.7^{**}	2.9^{+}
G x E	6	50	45	18	74	-4.1	0.7	-10.6	6.4	0.8	1.1	0.4^{*}	1.4
T x E	4	39	73	55	11	-5.9	7.5	1.3	-11.0	0.6	1.7	1.1	0.2
Heritibility		**0.92**	**0.94**	**0.92**	**0.77**								

$^{+}$, *, ** denotes significant at P=10, 5 and 1%

2.4.5 Genetic variation for spontaneous and Colchicine induced diploidisation rate of microspore derived embryos and for their direct embryo to plant conversion.

The previous experiments indicated a significant effect of the genotypes on the spontaneous and Colchicine induced diploidisation and on the direct embryo to plant conversion rate. Therefore, the following experiment was performed to characterise 17 different winter oilseed rape cultivars and F1-hyrbids for their spontaneous and Colchicine induced diploidisation and their direct embryo to plant conversion rate. The experiments were performed with Colchicine treatment (250 µM for 72 hours) and without.

The variance components showed a predominant effect of the Colchicine treatment on percentage of haploid, diploid and tetraploid embryos (Tab. 8). In contrast to this the variance components showed a predominant effect of the genotype on the direct embryo to plant conversion rate. The Colchicine treatment had no significant effect on the direct embryo to plant conversion rate. No significant correlation between diploidisation frequency with and without Colchicine and direct embryo to plant conversion could be detected (Spearman rank correlation, data not shown).

With Colchicine treatment the diploidisation rate of the plantlets ranged from 41.9% diploids for cultivar Krypton and 83.3% for cultivar Adriana (Tab. 9). 67.9% diploid embryos were obtained as a mean over all genotypes. Comparing cultivars and their F1-crosses, both achieved nearly the same percentage of diploids (69% vs. 68%; data not shown). Some F1-hybrids (Favorite × DSV1, Komando × Express 617, Charly × Krypton) led to an enhanced amount of diploids than their parental lines. For example, Komando x Express 617 resulted in 74.4% diploids, although the parental lines showed lower diploidisation frequencies of 62.5% (Komando) and 66.3% (Express 617). In contrast, other F1-crosses (Adriana x Oase, Express 617 × Charly, DSV1 × Charly) showed a reduced Colchicine induced diploidisation frequency compared to their parental lines. Surprisingly, whereas the parental lines DSV1 and Adriana led to 80.8 and 83.3% diploids, their F1-cross resulted in 70.8% diploids, only.

2.4.6 Effect of cultivars and their F1-crosses on spontaneous induced diploidisation frequency of microspore derived embryos

The mean of the spontaneous diploidisation frequency was 33.7% for all tested genotypes (Tab. 9). F1-crosses showed an increased number of diploids (37%) compared their parental lines (31%). The best frequency of doubled haploid plantlets was 69.3% (Favorite × DSV1),

whereas the lowest rate obtained was 14.9% diploids (Express 617). The spontaneous induced diploidisation frequency for some F1-crosses (Favorite × DSV1, Komando x Express 617, Charly × Krypton, Express 617 × Charly, Krypton × DSV2) indicated a higher percentage of diploids than their parental lines. For example, whereas the parental lines led to 22.8% (Favorite) and 30.1% (DSV1) diploids, their F1-cross (Favorite × DSV1) resulted in an increased number of spontaneous induced diploid plantlets. In contrast, other F1-crosses showed a reduced frequency compared to their parental lines. For example, DSV1 × Adriana resulted in 20.6% diploids, although their parental lines led to 30.1% (DSV1) and 58.1% diploids (Adriana). Interestingly, the F1-cross Adriana × Oase resulted in an intermediate frequency of diploids with 34% in contrast to the high (Adriana, 58.1%) and low (Oase, 17.3%) frequency of diploids of its parents.

The 17 cultivars and F1-crosses revealed a direct embryo to plant conversion rate (Tab. 4.2) ranging from 1.1% (Favorite × DSV2) to 46.7% (Express 617) without Colchicine treatment. The range with Colchicine treatment varied from 3.6% (Favorite × DSV2) to 39.7% (Krypton × DSV2). By comparison of Colchicine-treated F1-hybrids and their parental lines, the frequency of directly regenerated plantlets of the F1-crosses resulted in 16% and in 11% for the cultivars. Without Colchicine treatment, F1-hybrids resulted in 14% directly regenerated plantlets, whereas the frequency of the cultivars including their parental lines increased to 22%. Surprisingly, the cultivars Krypton, Komando, Express 617 and Adriana showed a reduced frequency of direct embryo to plant conversion (Tab. 9) by Colchicine treatment. For example, only 19.4% instead of 37.6% microspore derived embryos directly converted to plantlets when treated with Colchicine (250 µM for 72 hours). The F1-hybrids Adriana × Oase and Charly × Krypton showed similar results. Regarding these genotypes, the number of directly regenerated plantlets is clearly reduced by the standard Colchicine treatment. Furthermore, some F1-hybrids showed remarkable reduced frequencies of directly regenerated plantlets compared to their parental lines. Without Colchicine treatment, Komando × Express 617 achieved a very low rate of 6.3% directly regenerated plantlets whereas the parental lines resulted in 35.2% (Komando) and 46.7% (Express 617).

Tab. 8 Degrees of Freedom (DF), mean squares, variance components and F-values for ploidy level [%] and for direct plant conversion rate [%] of microspore derived embryos after Colchicine treatment

Source	DF	Mean squares				Variance components				F-value			
		Ploidy level			Direct embryo to plant conversion	Ploidy level			Direct embryo to plant conversion	Ploidy level			Direct embryo to plant conversion
		1x	2x	4x		1x	2x	4x		1x	2x	4x	
Genotype (G)	16	707	347	203	522	99.3	43.7	18.4	61.9	6.4**	4.1**	2.4*	3.5**
Treatment (T)	1	27383	11992	4013	175	530.4	228.5	78.3	1.6	82.6*	35.4*	200.1**	1.9
Experiment (E)	2	28	35	95	20	-3.7	-1.5	0.7	-1.1	0.2	0.4	1.3	0.3
G x T	16	342	151	93	150	62.7	21.8	11.5	21.0	2.2	1.8^{+}	2.4*	2.1*
G x E	32	111	84	20	94	-21.6	-0.6	-2.9	45.7	0.7	1.0	1.0	2.6**
T x E	2	331	339	70	59	10.4	14.9	70.1	2.1	2.2	4.0*	0.3	1.6
Heritability		**0.84**	**0.77**	**0.54**	**0.71**								

$^{+}$, *, ** denotes significant at P=10, 5 and 1%

Tab. 9 Doubled haploid plantlets rate [%] and direct plant conversion rate [%] of microspores derived embryos per genotype after Colchicine treatment and without Colchicine treatment. Depicted are means over three experiments.

	Treatment with and without Colchicine				
	Doubled haploid plantlets [%]		Direct embryo to plant conversion [%]		
Genotype	**With Colchicine treatment**	**Without Colchicine treatment**	**With Colchicine treatment**	**Without Colchicine treatment**	**Mean With/without Colchicine**
Favorite × DSV1	82.1	69.3	3.6	1.1	2.3
Favorite	61.8	22.8	3.2	2.1	6.4
DSV1	80.8	30.1	8.6	7.9	8.2
DSV1 × Adriana	70.8	20.6	27.9	25.4	26.7
Adriana	83.3	58.1	19.4	37.6	28.5
Adriana × Oase	60.9	32.8	14.6	23.5	19.0
Oase	75.8	17.3	8.7	13.7	11.2
Oase × NK Beauty	60.8	36.5	11.0	3.2	7.1
Komando × Express	74.4	45.8	4.8	6.3	5.6
Komando	62.5	37.9	12.8	35.2	24.0
Express 617	66.3	14.9	22.9	46.7	34.8
Express × Charly	61.6	32.9	14.6	11.4	13.0
Charly	70.5	21.6	22.5	29.4	26.0
Charly × Krypton	81.3	23.8	12.5	22.7	27.6
Krypton	41.9	23.6	9.2	23.0	16.1
Krypton ×DSV2	45.2	29.2	39.7	20.0	29.8
DSV2	73.9	55.7	10.1	2.7	2.6
Mean G	67.9	33.7	14.5	18.3	16.4
LSD5% G					17.6
LSD5% T	23.1		6.5		
LSD5% G x T	22.1		16.3		

2.5 Discussion

2.5.1 Spontaneous and antimitotic agent induced diploidisation of isolated microspore culture

In our study, the spontaneous diploidisation frequency showed a wide range from 14.9% to 69.3%, the mean over 17 genotypes was 33.7% (Tab. 9). In general, the spontaneous diploidisation rate of microspores derived plants is very low in rapeseed and varied from 10% to 30% (Möllers and Iqbal 2009). Hansen and Anderson (1996) reported 18% spontaneous chromosome doubling in *Brassica napus*. Klìma et al. (2008) reported for *Brassica carinata* a spontaneous diploidisation rate of 40%.

The results of this work showed that Colchicine induced diploidisation frequencies varied from 41.9% to 83.3%. The mean for all 17 genotypes was 67.9% (Tab. 9). Möllers and Iqbal (2009) reported that the diploidisation rate is genotype dependent and ranged from 50% to 70%. Colchicine treatment led to best mean diploidisation frequency of 77% in a concentration of 250 µM and a duration time of 48 hours. A duration time of 72 hours decreased the number of diploids. A longer duration time could lead to inhibitory side effects of the applied Colchicine. Hansen and Anderson (1996) showed the percentage of diploids increased with an increase of Colchicine concentration (3-1000 µM) and duration time (6-24 hours) but they also suggested that interactions between time and concentrations are mainly due to a higher toxicity of the antimitotic agent for microspore development. Toxic effects on microspore developments could be observed by Colchicine concentrations above 300 µM. The number of microspore derived embryos was decreased. Möllers et al. (1994) published that diploidisation frequencies of 80% to 90% were obtained after treating the microspores for 24 to 72 hours with 25 to 125 µM of Colchicine. Colchicine in a concentration of 125 µM for 24 hours and 25 µM for 72 hours resulted in the highest percentage of diploids. Their results showed that concentrations of 25 to 125 µM and duration time of 24 hours are needed to obtain 90% to 94% diploids.

In this study a concentration of 25 µM Colchicine incubated for 72 hours achieved a rate of 70.6% diploids. This treatment without subsequent washing of the microspores decreased the number of diploids (59.5%) and increased the number of tetraploids. Möllers et al. (1994) reported that the amount of tetraploids increased with the time of duration. A duration time longer than 72 hours would probably lead to a remarkable increase of

tetraploids. Pechan and Keller (1988) reported about the first embryogenic division of binucleate microspores after 48 hours, this indicate that binucleate microspores give rise to tetraploids and explain the occurrence of tetraploids at 72 hours of incubation.

Iqbal (1993) suggested a possible endoreduplication of the two nuclei through mitotic disruption by colchicine. Furthermore he constructed a “carry over” hypothesis: despite washing of the microspores an effective concentration of the antimitotic agent rests in the microspores, probably longer than 72 hours (Möllers et al. 1994). The diploidisation frequency could be more efficient with a duration time of 24 hours, by which time cell division of the uninucleate microspores had initiated (Iqbal 1993). The results of Möllers et al. (1994) and Hansen and Anderson (1996) confirmed that the majority of the first division of the microspores occurs within 24 hours. Pechan and Keller (2006) observed that *in vitro* cultured one-celled microspores introduce mitosis after approximately eight hours, while two-celled microspores enter mitosis after 24 hours. The diploidisation effect does not seem to be enhanced by treatments with a duration time of above 24 hours. Consequently, treatment durations of several hours up to 24 hours should be tested in further research. An efficient diploidisation frequency with a low Colchicine concentration of 25 µM without subsequently being removed and a duration time of several hours up to 24 hours could prove being practical and economical. Although the direct chromosome doubling of isolated microspores with Colchicine at a low concentration (25 µM) minimises chemical and disposal costs and reduces the risk of laboratory contamination, alternative antimitotic agents are needed having a higher affinity to plant tubulin and low toxicity. In this study, we investigated the effect of APM and Pronamide alone, in combination together, and with and without Colchicine on diploidisation of microspores and on the direct embryo to plant conversion rate of microspore derived embryos.

Results (Tab. 3) with the herbicides APM and Pronamide disproved the assumption both herbicides combined may enhance the ability of chromosome doubling. A synergistic mode of action was not detected. The combination of Pronamide, APM and Colchicine showed also no synergistic effect on diploidisation frequency. The three antimitotic agents increased the rate of tetraploids. APM in a concentration of 3 µM decreased the rate of diploids. The rate of diploid plantlets was lower than without antimitotic treatment. No antimitotic mode of action was detected. Results published by Hansen and Anderson (1996) were not confirmed. In their study they reported of a diploidisation rate of 60% by treatment with APM in a

concentration of 3 µM. In contrast to this study, the duration time was 12 hours instead of 72 hours. Hansen and Anderson (1996) observed toxic effects of APM with concentrations above 300 µM and a duration time longer than 24 hours. In previous single experiments (no statistical calculation, data not shown) no increased diploidisation rate by APM treatment with a duration time of 12 and 48 hours instead of 72 hours was observed.
Pronamide led to 52.0% diploids. Several genotypes (Charly × Krypton, Krypton × DSV2) resulted in diploidisation rates of 74.7% and 82.5% (Tab. 3). In comparison, Charly × Krypton achieved 81.3% diploids by standard Colchicine treatment (250 µM, 72hours) and Krypton × DSV2 45.2% (Tab. 3). For several genotypes, Pronamide seems to be an antimitotic alternative to Colchicine with less toxic effects. Hanson et al. (1998) postulated that Pronamide (0-300 µM) had no significant toxic effect for the following development of the microspores but it induced chromosome doubling at lower frequencies in *Beta vulgaris.*

2.5.2 Effect of antimitotic agents on embryogenesis

It is already known that the response of microspore culture is highly genotype dependent (Ferrie et al. 2005). Several genotypes (Adriana × Oase, DSV1 × Adriana, and Adriana) showed a low embryogenesis response with 20 to 50 embryos per Petri dish. Other genotypes (Komando × Express, Charly × Krypton) showed a very high embryogenesis response with about 1000 embryos per Petri dish. Next to the genotype, the antimitotic agent seems to influence the embryogenesis.
By comparison to the untreated control, we observed the highest embryogenesis after previous Colchicine treatment with 250 µM (48 hours and 72 hours incubation) and 25µM (72 hours incubation) for all tested treatments and genotypes. Zaki and Dickinson (1991) suggested that Colchicine induced the symmetrical division of microspores, which diverts microspores from gametophytic to sporophytic development. Chen et al. (1994) published that the microspore treatment with Colchicine stimulated embryogenesis for most of the tested lines. Iqbal et al. (1994) reported that the concentration of 250 µM Colchicine (72 hours incubation) led to the highest increase in embryogenesis. By comparing the treatments of 25 µM Colchicine incubated for 72 hours and a permanent incubation (without removal of the Colchicine), we observed a reduced embryogenesis in the Petri dishes by 25 µM treatment with permanent incubation. To obtain higher embryogenesis the Colchicine has to be removed by a media change. Regarding all genotypes, a clearly

decreased embryogenesis was observed by APM treatment (3 µM). Dependent from the genotype, the 3 µM APM microspore treatment resulted in 25 to 60 embryos per Petri dish in contrast to Colchicine treatment resulting in 150 up to 1000 embryos per Petri dish. Zhao and Simmonds (1995) observed a negative effect on embryogenesis based on the slow recovery rate of the microtubules after treatment with several antimitotic agents (1-10 µM). They reported that fewer microspores develop into embryos; the result is a reduced embryogenesis.

Results of this study showed that APM in a concentration of 3 µM decreased the embryogenesis while Colchicine in a concentration of 250 µM mainly increased the number of microspore derived embryos. Pronamide in a concentration of 1.5 µM and 3 µM had no effect on embryogenesis.

2.5.3 Effect of DMSO in addition to Colchicine on diploidisation and direct embryo to plant conversion of microspore derived embryos

In general, DMSO is used as a plant cryoprotectant. Vannini and Poli (1983) reported about the effect of DMSO on tubulin protein in microtubules resulting in chromosome doubling. Chauvin et al. (2003) had the assumption that DMSO in combination with Colchicine may act synergistically to induce doubling of chromosomes in *Solanum* species.

In this study, the antimitotic agent Pronamide and APM were dissolved in DMSO and directly applied to microspore culture. The final concentration of DMSO was 0.3%. Up to date the effect of DMSO on embryogenesis, diploidisation and direct embryo to plant conversion of microspore derived embryos still remains unclear. High concentrations of DMSO may inhibit the embryogenesis. Subrahmanyam and Kasha (1975) postulated that 3% DMSO acts as a Colchicine carrier and promotes the antimitotic mode of action. We analysed the influence of DMSO in combination with the antimitotic agent Colchicine. An effect of DMSO on embryogenesis could not be detected. Furthermore, no significant effect of DMSO on ploidy level and direct embryo to plant conversion was determined.

2.5.4 Effect of antimitotic agents on direct embryo to plant conversion of microspore derived embryos

The ability of microspore derived embryos to regenerate directly to plants seems to depend mainly on the genotype in *Brassica napus*. The observed genotypic influence on direct embryo to plant conversion in this study confirms results postulated by several studies (Kott

and Beversdorf 1990, Baillie et al. 1992, Mathias 1988). In *B. napus* the frequencies of plantlet development varied from 1% to 47% (Kott and Beversdorf 1990). In this study, by comparison of 17 different genotypes including eight cultivars and nine derived F1-hybrids the direct embryo to plant conversion rate varied from 2.3% to 34.8% (Tab. 9). The Colchicine treatment showed no significant effect on direct embryo to plant conversion rate (Tab. 8), the frequency of directly converted microspore derived embryo to plantlets was not significantly different (LSD 5% 6.5) in the treatments with (mean 14.5%) and without Colchicine (mean 18.3%). The treatment with the antimitotic agents APM and Pronamide also had no significant effect on direct embryo to plant conversion rate (Tab. 2). These results correspond with publications by Hansen and Anderson (1996) and Klíma et al. (2008).

2.5.5 Searching for novel antimitotic agents

Next to Pronamide and APM, alternative antimitotic agents Oryzalin and Trifluralin were tested in literature. Hansen and Anderson (1996) stated that both agents showed an efficient diploidisation frequency but also an inhibiting effect on embryo formation. For that reason high throughput-screening of chemical libraries is necessary to detect novel mitotic inhibitors which are efficient and less toxic for human beings. Häntzschel (2011) showed that the LATCA-library using a transgenic *Arabidopsis thaliana* line was very effective for screening mitotic inhibitors. After screening of 3600 chemicals and with the help of experiments with *Arabidopsis thaliana,* 51 chemicals could be detected as potential mitotic inhibitors. Further investigations are needed to understand mechanisms in haploid induction. This information could be helpful to choose a specific antimitotic agent for doubled haploid production.

In general, next to the antimitotic agent Colchicine, stress is considered to be inducing embryogenesis in microspores. By stress application of the donor plants or isolated microspores the microspores follow the sporophytic pathway instead of the normal gametophytic pathway to form pollen grains (Ferrie and Caswell 2011). Shariatpanahi et al. (2006) gave an overview about widely used, neglected, and novel stress applications. Cold/heat, sugar starvation, and Colchicine treatment are widely used stress inducers, while γ-irradiation, ethanol stress, hypertonic shock, centrifugal treatment, reduced atmospheric pressure, feminizing agents, and phytohormone Abscisic Acid (ABA) were considered neglected stresses. Very interesting for further research in *Brassica* could be novel stress

factors including high medium pH, carrageenan oligosaccharide, heavy metal stress, inducer chemicals, and a 2,4D pre-treatment.
Putative stress inducers may further improve the spontaneous or antimitotic induced diploidisation frequency.
Furthermore, efficient chromosome doubling methodologies in other plant species (cereal or fruit and vegetable species) could be helpful to find new doubling agents for application in *Brassica* species. For example, in wheat, the primary alcohol *n*-butanol, known as an activator of phospholipase D was successfully applied in the production of wheat doubled haploids (Soriano et al. 2008). By application of *n*-butanol, the doubled haploid plant production could be remarkably increased. The agent *n*-butanol seems to disrupt cortical microtubules and detaches them from the plasma membrane in plants (Hirase et al. 2006).
A number of alternatives exist which may have the potential to replace an application of Colchicine in doubled haploid production in *Brassica napus*. Nevertheless, further research is required to attain an efficient diploidisation frequency with and without the antimitotic agent Colchicine.

2.6 Conclusion

This study has shown that no synergistic effect on diploidisation frequency by combination of antimitotic agents could be detected. An efficient antimitotic induced diploidisation frequency of about 90% could not be achieved. Nevertheless, the highest induced mean diploidisation of 77% could be determined by Colchicine treatment with 250 µM incubated for 48 hours. A concentration of 25 µM incubated for 72 hours led to 70% diploids. A permanent incubation of Colchicine without a media change was also successful (60%) and not replacing the media is time and cost-saving in a plant breeding program.
A less toxic alternative to Colchicine could be Pronamide resulting in over 50% diploids. Pronamide showed no negative effect on embryogenesis and on direct embryo to plant conversion frequency of microspore derived embryos. Pronamide applied in a higher concentration (>3 µM) could also enhance the induced diploidisation frequency.
The results of this study indicate that a wide variation for the spontaneous diploidisation frequency exists in *Brassica napus*. Furthermore a selection for genotypes with a “high

diploidisation frequency” is feasible. By inheritance of the trait the diploidisation frequency could be improved step by step.

Further investigations are necessary to attain an efficient diploidisation frequency without the high toxic antimitotic agent Colchicine and to study the effect of the ploidy level on the direct embryo to plant conversion rate.

2.7 Acknowledgments

The technical assistance of Rosemarie Clemens and Carmen Mensch is greatly appreciated. The authors gratefully acknowledge the Federal Ministry of Economy & Technology and the Gemeinschaft zur Förderung der Privaten deutschen Pflanzenzüchtung (GFP e.V.) for funding this research project (ÖE 134/09 NR).

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Chapter 3:

Effect of shoot regeneration media, phytohormone and cold treatments on direct embryo to plant conversion of microspore derived embryos from different winter oilseed rape genotypes

3.1 Abstract

In microspore culture of oilssed rape (Brassica napus L.) the direct embryo to plant conversion is hampered by the occurrence of secondary embryogenesis and callus development. Laborious, time and cost intensive sub-culturing of those structures is often required before normal shoots are obtained. The objective of the present study was to improve the direct embryo to plant conversion.

The present research, including two experiments, was started to analyse the effect of different shoot regeneration media with and without the phytohormone gibberellic acid (GA_3), 6-benzylaminopurine (BAP), and indole-3-butyric-acid (IBA). Additionally, the experiment investigated the effect of cold storage for a period of 14 days at temperatures at 1.5 °C and 4 °C linked with different photoperiods (eight hours and continuous light) and without light on the embryo to plant conversion of different winter oilseed rape genotypes. In the first experiment, microspore derived embryos of five winter oilseed genotypes were cultured on the Murashige and Skoog and the Gamborg B5 media supplemented with and without various concentrations and combinations of GA_3, IAA and BAP at 4 °C under 24 hours and eight hours light for 14 days, compared to standard conditions (26 °C, 12 hours light). The five genotypes showed a range from 13% to 39% direct embryo to plant conversion rate. Microspore derived embryos cultivated on B5 medium with 0.1 mg/L GA_3 resulted in best frequency of about 43%, while cultivation on B5 with 0.1 mg/L GA_3 and 0.2 mg/L BAP resulted in the lowest rate of 11% direct embryo to plant conversion. The two-week cold treatment at 4 °C significantly increased the mean for direct embryo to plant conversion rate from 14% (standard conditions) to 28%.

In the second experiment, microspore derived embryos of 13 winter oilseed genotypes were cultivated on the four best culture media (MS, B5, and B5 with 0.1 mg/L GA_3, B5 with 0.1 mg/L IBA and with 0.2 mg/L BAP) and exposed to 1.5 °C and 4 °C for 14 days compared to

standard conditions. The temperature 1.5 °C was linked with eight hours light and continuous darkness. In contrast to the first experiment, the temperature at 4 °C was linked with continuous light and darkness. The genotypes including cultivars and F1-hybrids showed a wide range from 29% to 76% direct embryo to plant conversion frequency. The variation of the different culture media ranged from 50% (MS) to 60% (B5 with 0.1 mg/L GA_3) and for the culture conditions from 21% (standard conditions) to 71% (LT 1.5 °C continuous darkness).The genotypes Krypton and Krypton × DSV2 obtained 100% direct embryo to plant conversion rate after cultivation of microspore derived embryos on B5 with 0.1 mg/L GA_3 and a two-week cold treatment at 1.5 °C under continuous darkness. This study showed that although the ability of microspore derived embryos to convert directly to plantlets is genotype depended; two-week cold treatment at 1.5 °C and at 4 °C with and without light significantly increased the direct embryo to plant conversion rate, while the choice of the shoot regeneration media plays an subordinated role in direct embryo to plant conversion.

3.2 Introduction

Microspore culture is being used routinely in oilseed rape breeding programs for the production of doubled haploid lines. However, the efficient conversion of microspore derived embryos to plants still represents a bottleneck in the procedure. With *B. napus* frequencies of direct embryo to plant conversion ranged from 1% to 47% (Kott and Beversdorf 1990), while with *B. campestris (B.rapa)* the range varied from 5% to 20% (Baillie et al. 1992, Burnett et. 1992). In more recent previous work, direct embryo to plant conversion rates ranging from 1% to 47% were observed for different winter oilseed rape cultivars (see Chapter 2, this work). The embryos which do not directly convert to plants are either not at all responding, or they produce callus or they undergo secondary embryogenesis (Hays et al. 2001, Ferrie and Caswell 2011). The plantlet regeneration from callus and secondary embryos requires multiple sub-culturing of the tissues which is laborious, time and cost intensive.

During microspore derived embryo to plantlet conversion the embryo passes through globular, heart-shaped, torpedo, and cotyledonary developmental stages which are similar to zygotic embryo development (Ilić-Grubor et al. 1998). After 28 to 35 days of culture microspore derived embryos reach the cotyledonary stage of development showing an

elongated root/shoot axis and laterally placed cotyledons (Senaratna 1992). Embryos at this developmental stage transferred to solid media showed the highest occurrence of direct embryo to plant conversion (Burnett et al. 1992). The regeneration of embryo to well-developed plantlets depends on a number of influencing factors. Next to the developmental stage of the embryo and embryo age, the shoot regeneration media, culture conditions, and the genotype are also playing an important role in the enhancement of the direct conversion of microspore derived embryos to plantlets (Huang et al. 1991, Kott and Beversdorf 1990, Tian et al. 2004, Belmonte et al. 2006, Haddadi et al. 2008, Ferrie and Caswell 2011).

The choice of shoot regeneration media with and without phytohormones seems to significantly influence the efficiency of direct conversion of microspore derived embryos to plantlets. B5 medium without any growth regulators (Gamborg et al. 1968, Ferrie et al. 2005) and MS medium (Murashige and Skoog 1962, Zhou et al. 2002) are often used for cultivation of microspore derived embryos. In various studies, B5 medium supplemented with 0.1 mg/L gibberellic acid (GA_3) is used for germination induction of the embryos (Kott and Beversdorf et al. 1990, Coventry et al. 1988, Fletcher et al. 1998, Cegielska-Taras et al. 2002). GA_3 is usually added in plant tissue to stimulate stem elongation. Hays et al. (2001) examined the role of gibberellins during early embryo development of *Brassica napus*. Next to the phytohormone GA_3, 6-benzylaminopurine (BAP) was applied for shoot development and multiplication (Tang et al. 2003). With regard to the best culture media for embryo to plant conversion some reports (Gland-Zwerger 1995, Zhang et al. 2006) announced that shoot regeneration media supplemented with 2.0 mg/L BAP had shown efficient direct embryo to plant conversion. Auxins are also often applied in *in vitro* culture. Besides naphthaleneacetic acid (*NAA*) and indoleacetic acid (IAA), indole-3-butyric-acid (IBA) also belonging to the auxin-familiy is known to stimulate extension growth and root building. Zhang et al. (2006) reported that the combination of 0.1 mg/L BAP and 0.2 mg/L IBA led to an efficient percentage of haploid plant production. Growth regulator type and composition can influence frequencies of direct embryo to plant conversion.

Related to the role of culture conditions, results from other studies indicate that a short term cold treatment of the microspore derived embryos may enhance their conversion to plantlets (Kott and Beversdorf 1990, Fletcher et al. 1998, Yeung et al. 1996, Cegielska-Taras et al. 2002, Zhou et al. 2002, Gu et al.2004, Zhang et al. 2006). Cegielska-Taras et al. (2002) reported of the best obtained embryo conversion rate of over 70% at 1 °C, compared to

about 20% at 4 °C for 14 days with a short photoperiod (8 hours light/day). In contrast, Zhang et al. 2006 postulated an improved direct embryo to plant conversion after three to five days cold treatment at 4 °C with a long photoperiod (16 hours light/eight hours dark). Referring to literature, in our study, the effect of the photoperiod on direct embryo to plant conversion of microspore derived embryos is still unclear. It is also unclear if light is required for efficient direct conversion of microspore derived embryos to plantlets.

Additionally, several studies showed that the ability of microspore derived embryos to convert directly to plantlets is depending on the genotype (Chuong et al. 1988, Kontowski and Friedt 1994).

Over the past few years, there have been many research reports about improvements in microspore isolation and regeneration in *Brassica* species. Nevertheless, there is still a need of advancement of microspore isolation and regeneration systems to provide the opportunity of producing large numbers of plants with minimal effort and technical resources. In this study we investigated three effects: shoot regeneration media with and without phytohormones (BAP, GA_3, IBA), cold treatment linked with different photoperiods and the effect of the genotype on the development of plantlets of microspore derived embryos of winter oilseed rape cultivars and F1-hybrids.

3.3 Materials & Methods

3.3.1 Donor plant material

Microspore donor plants of winter oilseed rape cultivars Charly, DSV1, DSV2 , Krypton, Oase and F1-donor plants of the crosses Komando × Express, Express × Charly, Charly × Krypton, Krypton × DSV1, DSV1 × Adriana, Adriana × Oase, Oase × NK Beauty and Favorite × DSV2 were used for the experiments. F1-donor plants were clonally propagated *in vitro* and plantlets were transferred to soil whenever donor plant material was needed.

3.3.2 Donor plant growth

See Materials & Methods Chapter 2.

3.3.3 Bud selection and microspore culture

See Materials & Methods Chapter 2.

3.3.4 First series of experiments

After three to four weeks of culture, cotyledonary microspore derived embryos at the age of 20 to 27 days after microspore isolation and a size of 7 mm to 10 mm were transferred to agar (1%, Plant Agar, C.E. Roeper GmbH, Germany) solidified Gamborg B5 and Murashige & Skoog media with and without the phytohormones gibberellic acid (GA_3), indole-3-butyric acid (IBA) and 6-benzylaminopurine (BAP). Basal media and phytohormones were obtained from Duchefa, Haarlem, The Netherlands. The following variants of culture media were tested: B5, B5 with 0.1 mg/L GA_3, B5 with 0.1 mg/L IAA and 0.2 mg/L BAP, B5 with 0.1 mg/L GA_3 and 0.1 mg/L IAA and 0.2 mg/L BAP, B5 with 0.1 mg/L GA_3 and 0.1 mg/L IAA, B5 with 0.1 mg/L GA_3 and 0.2 mg/L IAA, B5 with 0.1 mg/L GA_3 and 0.1 mg/L BAP, B5 with 0.1 mg/L GA_3 and 0.2 mg/L BAP, MS and MS with 0.1 mg/L IAA and 0.2 mg/L BAP in disposable plastic boxes (10.5 x 8x 5cm, Volume 0.24 L Huhtamaki GmbH & Co.KG, Alf, Germany). Each box contained 25 mL media and eight microspore derived embryos. Boxes with embryos were incubated in two Light Thermostats (LT, Rumed) adjusted to 4 °C, one with continuous light (24 h, Osram Cool white) and the other with eight hours (8 h) light. After 14 days of incubation to reduce secondary embryogeneis (Kott and Beversdorf 1990, Fletcher et al. 1998, Zhang et al. 2006), boxes were transferred for another four weeks to the culture room (26 °C). For comparison, boxes with microspore derived embryos were also incubated for six weeks under standard conditions in a culture room (CR) at 26 °C and 12 hours light (Osram Cool white). Three experiments with five genotypes were performed with each eight microspore derived embryos per experiment and treatment. After a total of six weeks the direct embryo to plant conversion was scored per plastic box in all treatments. In the three experiments altogether 720 embryos per genotype (1 genotype x 10 shoot regeneration media x 3 culture conditions x 8 microspore derived embryos x 3 experiments) were scored for their direct embryo to plant conversion rate.

3.3.5 Second series of experiments

With microspore derived embryos of the same age and size as in the previous experiments the following variants of culture media were tested: B5, B5 with 0.1 mg/L GA_3, B5 with 0.1 mg/L IAA and 0.2 mg/L BAP and MS in disposable plastic boxes (10.5 x 8 x 5 cm, Volume 0.24 L Huhtamaki GmbH & Co.KG, Alf, Germany). Each box contained eight microspore derived embryos in 25 mL media. Boxes with embryos were incubated in two Light Thermostats (LT,

Rumed), one adjusted to 1.5 °C and the other one to 4 °C with continuous light (24 h, Osram Cool white) and in continuous darkness (0h). For comparison, boxes with microspore derived embryos were also incubated under standard conditions in a culture room (CR) at 26 °C and 12 hours light (Osram Cool white) and an incubation period of six weeks. Three experiments were performed with each eight microspore derived embryos per genotype and treatment. After 14 days of incubation at low temperatures, boxes were transferred to the CR (26 °C) and after four weeks the direct embryo to plant conversion was scored. The experiments were performed with 13 genotypes. In the three experiments 480 embryos per genotype (1 genotype x 4 shoot regeneration media x 5 culture conditions x 8 microspore derived embryos x 3 experiments) were scored for their direct embryo to plant conversion.

3.3.6 Direct embryo to plant conversion

The direct embryo to plant conversion was scored per plastic box containing eight embryos after four (after cold induction in Light Thermostats) to six weeks (control) under standard conditions (CR, 26 °C and 12 hours light).

3.3.7 Analysis of variance

Depending on the experiment between five and 13 genotypes were used in combination with three to five different culture conditions and four to ten different shoot regeneration media. Per genotype, culture condition and shoot regeneration media, three independent experiments were performed, and in each experiment percentage of direct embryo to plant conversion was determined using eight microspore derived embryos. A two-way analysis of variance (ANOVA) was performed as series of experiments (E=3) over locations (culture conditions (C)) with the factors 'genotype' (G), and 'shoot regeneration media' (M) by using PLABSTAT software (Utz 2011) considering the genotypes and experiments as random. ArcSin-transformed data were used in the ANOVA and significant differences between genotypes, treatments and interactions were assessed by LSD calculated at $\alpha=0.05$ confidence level.

3.4 Results

3.4.1 Effect of a 4 °C cold treatment under continuous and eight hours light and of different culture media on the direct embryo to plant conversion of different winter oilseed rape genotypes

The following experiment was performed to improve direct embryo to plant conversion of microspore derived embryos. The effect of ten different shoot regeneration media with and without phytohormones GA_3, BAP and IBA and a 14-day cold treatment of microspore derived embryos at 4 °C under eight and 24 hours light/day were studied. The analysis of variance showed highly significant effects of the genotypes, the shoot regeneration media and the culture conditions on direct embryo to plant conversion of five winter oilseed rape genotypes (Tab. 10). The variance components revealed a predominant effect of the genotypes, followed by the effect of the shoot regeneration media and the culture conditions. There were also significant interactions between genotypes and media and genotypes and culture conditions. Heritability for the trait direct embryo to plant conversion was high with 0.99.

Tab. 10 Degrees of Freedom (DF), mean squares, variance components and F-values for direct embryo to plant conversion rate [%] of microspore derived embryos after their incubation on ten different shoot regeneration media and under three different culture conditions

Source	DF	Mean squares	Variance components	F-value
Genotype (G)	4	10431	114.0	60.1^{**}
Media (M)	9	2575	54.6	21.4^{**}
Experiment (E)	2	281	1.2	2.9^{+}
Culture conditions (C)	2	5837	38.2	58^{**}
G x C	8	2696	85.7	21.5^{**}
G x E	8	150	2.5	1.5^{*}
G x M	36	710	62.4	4.8^{**}
M x C	18	260	12.5	3.6^{**}
M x C x G	72	379	93.6	3.9^{**}
Heritability			**0.99**	

$^{+}$, *, ** **denotes significant at P=10, 5 and 1%**

The mean of the ten different shoot regeneration media (Fig. 1) ranged from 10.8% (B5 + 0.1 mg/L GA_3 + 0.2 mg/L BAP) to 43.0% (B5 + 0.1 mg/L GA_3). B5 media without phytohormones and B5 media with 0.1 mg/L GA_3, 0.1 mg/L IBA and 0.2 mg/L BAP led to 30.0% directly regenerated plantlets. By cultivation of microspore derived embryos on B5 with GA_3 (0.1 mg/L) and IBA in a concentration of 0.1 mg/L and 0.2 mg/L medium about 19.2% direct embryo to plant conversion rate was achieved. Microspore derived embryos cultivation on MS media achieved an average of 24.7%. B5 and MS media with the phytohormones IBA (0.1 mg/L) and BAP (0.2 mg/L) obtained a mean of 17.7% and 17.3%.

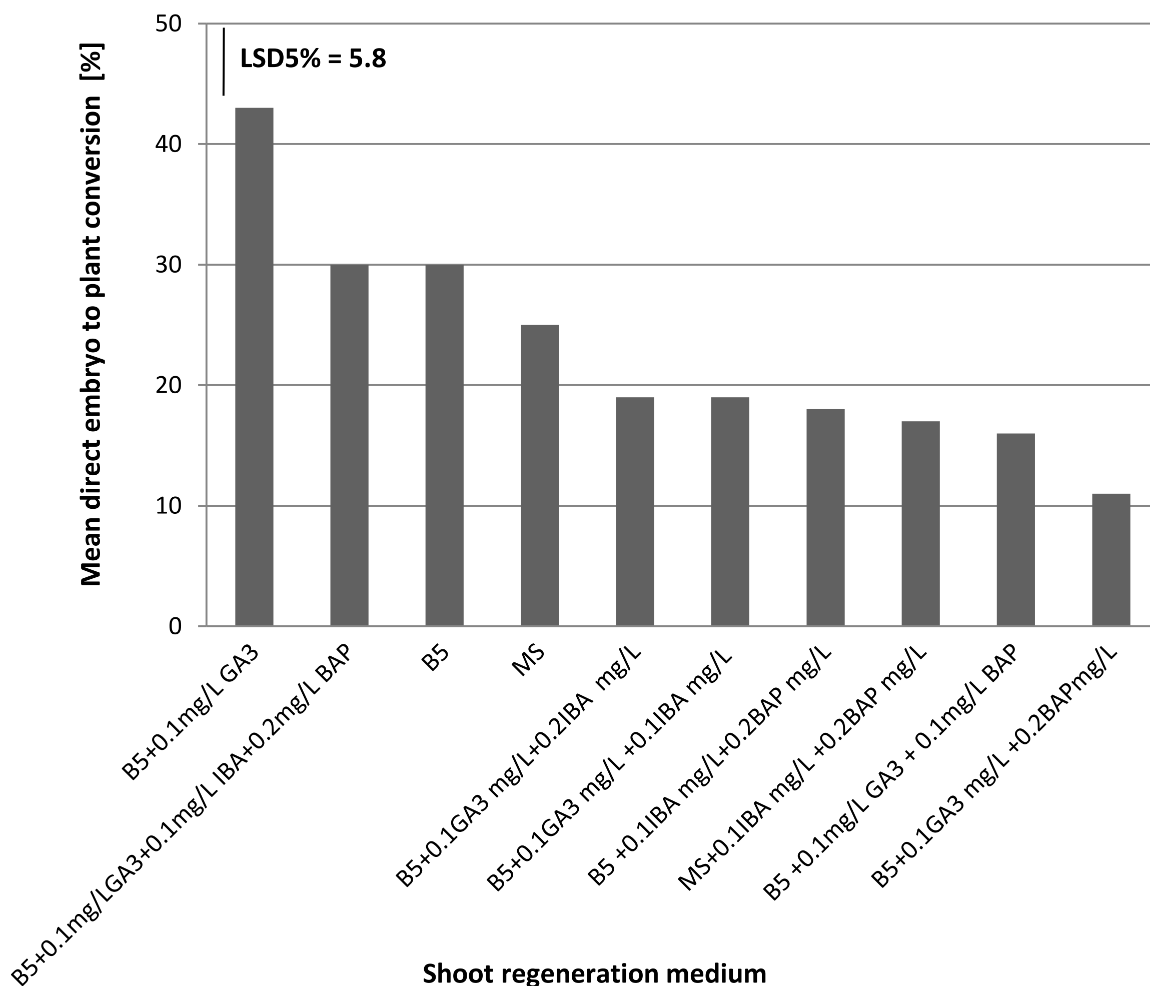

Fig.1 Direct embryo to plant conversion rate of microspore derived embryos cultivated on ten shoot regeneration media. Depicted are means over five genotypes, three culture conditions and three experiments.

In addition, the mean direct embryo to plant conversion rate of the three examined culture conditions ranged from 14.1% for the control conditions (CR 26 °C, 12 h light) to 27.9% for cold treatment (LT 4 °C, 24 h light treatment). At 4 °C and eight hours light a direct embryo to plant conversion rate of 26.1% was obtained. The continuous light treatment at 4 °C showed an equally remarkable positive effect on the direct embryo to plant conversion rate (Fig. 2).

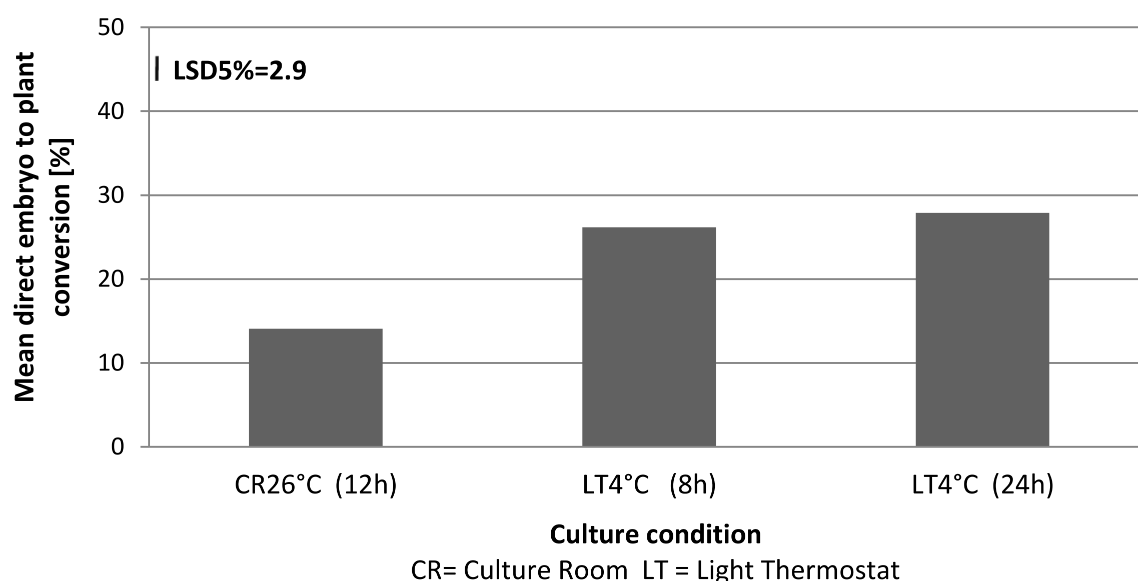

Fig.2 Direct embryo to plant conversion rate of microspore derived embryos cultivated under three culture conditions. Depicted are means over five genotypes, ten shoot regeneration media and three experiments.

The significant interaction between genotypes and media (Tab. 10) indicated that the genotypes responded differently to the culture conditions. Compared to standard conditions both cold treatments had to varying degrees positive effects on the direct embryo to plant conversion rates (Tab. 11) for nearly all tested genotypes with the exception of the cross Komando × Express, where the 4 °C cold treatment at eight hours light led to a reduced direct embryo to plant conversion rate in comparison to the control treatment.

Tab. 11 Direct embryo to plant conversion rate of microspore derived embryos per genotype cultivated under three culture conditions. Depicted are means over ten shoot regeneration media and three experiments.

	Direct embryo to plant conversion rate [%]			
		Culture condition		
Genotype	**Mean**	**CR 26 °C (12 h)**	**LT 4 °C (8h)**	**LT 4 °C (24 h)**
Krypton × DSV2	39.2	8.3	53.3	55.9
Krypton	32.5	27.1	36.7	33.8
Komando × Express	13.4	20.3	19.1	12.1
Oase × NK Beauty	17.2	7.9	13.7	18.7
Charly ×Krypton	11.3	6.7	16.8	10.4
Mean	22.8	14.1	26.2	27.9
LSD5% G	4.8			
LSD5% C			2.9	
LSD5% G x C			5.6	

Although the B5 media with 0.1 mg/L GA_3 on average resulted in the best direct embryo to plant conversion rate (Fig. 1) across all genotypes and culture conditions, the genotype Krypton (Tab. 12) proved to be an exception. The B5 media with GA_3, IBA and BAP in combination achieved the highest mean (77.8%) across three culture conditions and three experiments. In contrast, by cultivation of microspore derived embryos on B5 with GA_3 and B5 with GA_3 and IBA (0.1 mg/L and 0.2 mg/L) no distinct difference on average of directly regenerated plantlets was obtained. The results also implicate a strong influence of the genotype concerning which examined shoot regeneration media shows the best performance to enhance the direct conversion of microspore derived embryos to plantlets. The observed interaction of genotype, shoot regeneration media and culture conditions influenced the resulting direct embryo to plant conversion rate of microspore derived embryos.

Tab. 12 Direct embryo to plant conversion rate of microspore derived embryos per genotype cultivated on ten different shoot regeneration media. Depicted are means over three culture conditions and three experiments.

	Direct embryo to plant conversion rate [%]										
	Shoot regeneration medium										
Genotype	**Mean**	**B5 +0.1mg/L GA_3**	**B5 +0.1mg/L GA_3 +0.1mg/L IBA +0.2mg/L BAP**	**B5**	**MS**	**B5 +0.1mg/L GA_3 +0.2mg/L IBA**	**B5 +0.1mg/L GA_3 +0.1mg/L IBA**	**B5 +0.1mg/L IBA +0.2mg/L BAP**	**MS +0.1mg/L IBA +0.2mg/L BAP**	**B5 +0.1mg/L GA_3 +0.1mg/L BAP**	**B5 +0.1mg/L GA_3 +0.2mg/L BAP**
Krypton × DSV2	39.2	58.3	46.9	47.2	51.1	43.1	29.2	36.2	29.6	25.0	25.0
Krypton	32.5	52.8	77.8	33.3	23.6	33.3	25.0	13.9	19.4	33.3	12.5
Komando ×Express	13.4	37.5	1.4	18.1	18.1	13.9	6.9	8.2	15.3	9.7	5.4
Oase × NK Beauty	17.2	35.8	7.2	22.2	16.4	4.2	28.4	25.8	9.7	11.1	11.1
Charly ×Krypton	11.3	30.5	16.7	27.8	14.3	1.4	4.4	4.2	12.5	1.4	0.0
Mean	22.8	42.9	30.0	29.7	24.7	19.2	18.8	17.7	17.3	16.1	10.8
LSD5% G	4.8										
LSD5% M							5.8				
LSD5% G x M							13.1				

3.4.2 Effect of a 4 °C and a 1.5 °C cold treatment with and without light and of different culture media on the direct embryo to plant conversion of different winter oilseed rape genotypes

The previous experiment showed that a 14-day cold treatment at 4 °C has a positive effect on the direct embryo to plant regeneration rate. The present experiment was performed to analyse the effect of an even lower incubation temperature (1.5 °C) on the direct embryo to plant regeneration rate. Since temperatures lower than 4 °C in the presence of light are not always easy to realise, experiments were not only performed under light (8 h and 24 h) but also in darkness. Furthermore, the effect of the four best shoot regeneration media from the previous experiment was analysed.

In agreement with the results from the first experiment, the analysis of variance indicated highly significant effects of the genotype, the media and the culture conditions on the direct embryo to shoot conversion (Tab. 13). In contrast to the first experiment (Tab. 10), predominant effects of culture conditions, followed by the genotypes were revealed by the variance components. The efficiency of microspore derived embryos directly converted to plantlets depended on the choice of culture condition which was more important than the choice of the genotype. The factor shoot regeneration medium was subordinated. The interaction effect for genotype and culture conditions was also significant. For the trait direct embryo to plant conversion a high heritability of 0.99 was obtained.

Tab. 13 Degrees of Freedom (DF), mean squares, variance components and F-values for direct embryo to plant conversion rate [%] of microspore derived embryos after their incubation on four different shoot regeneration media under five different culture conditions

Source	DF	Mean squares	Variance components	F-value
Genotype (G)	12	9565	157.2	71.4**
Media (M)	3	3040	14.5	14.1**
Experiment (E)	2	33	-0,2	0.4
Culture conditions (C)	4	32000	203.0	96.9**
G x C	48	821	59.6	7.8**
M x C	12	293	4.5	3.3**
M x G	36	53	-2.3	0.6
M x C x G	144	172	26.1	1.8**
Heritability			**0.99**	

** denotes significant at P=1%

3.4.3 Effect of the genotype, best shoot regeneration media and cold treatment with and without light on direct embryo to plant conversion

In the first experiment the B5 with 0.1 mg/L GA_3 medium proved to be the best culture medium for direct embryo to shoot conversion (Fig. 3). Furthermore, the positive results from the cold treatment could be confirmed. The standard cultivation in culture room at 26 °C under 12 hours light/day led to a low average direct embryo to plant conversion rate of 21.6% (Fig. 3 and Tab. 14). As a mean over all 13 genotypes and four culture media, the two week cold treatment of microspore derived embryos at 1.5 °C without light resulted in the highest direct embryo to plant conversion rate of 71.4% .

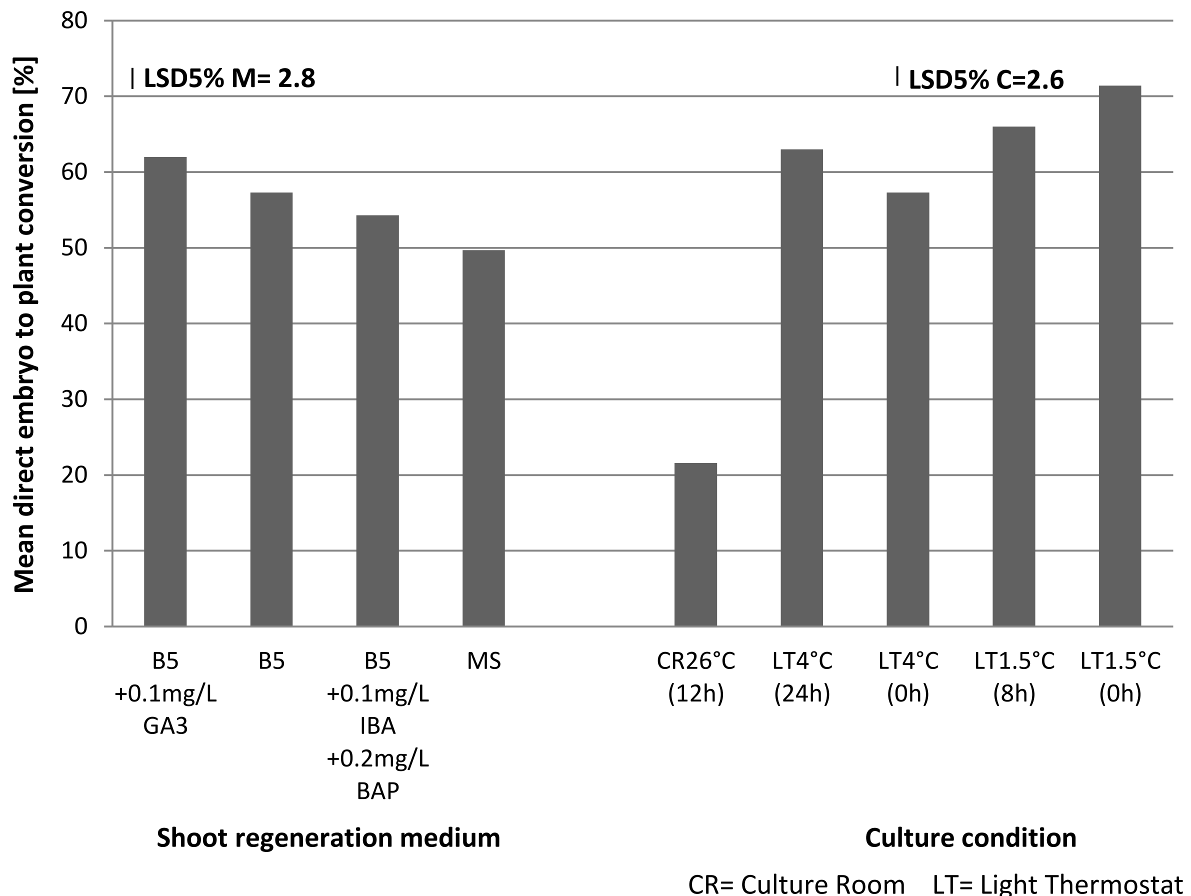

Fig. 3 Direct embryo to plant conversion rate of microspore derived embryos cultivated under four different shoot regeneration media (left) and on five culture conditions (right). Depicted are means over thirteen genotypes, three experiments and five culture conditions or four media, respectively.

The 13 winter oilseed rape genotypes (Tab. 2.2) showed a mean direct embryo to plant conversion rate ranging from 29.4% (Charly × Krypton) to 78.7% (Krypton). Although the absolute values for direct shoot regeneration rate for the five genotypes tested in the first and the second experiment were quite different (compare Tab. 1.1 and Tab. 2.2) the same responses with respect to culture conditions and media were observed. The best culture condition in combination with the best culture media as a mean over all genotypes resulted in 71% direct embryo to plant conversion rate (Tab. 2.2). For example, Krypton × DSV2 obtained the highest frequency of directly regenerated plantlets of 100%. The interaction of culture condition and genotype following shoot regeneration media influenced the direct embryo to plant conversion frequency of microspore derived embryos.

The cultivars Oase × Charly and the F1-crosses Express 617× Charly, Charly × Krypton and DSV1 resulted in very low frequencies for direct embryo conversion (Tab. 2.2), whereas for Express 617 × Charly and DSV1 the cultivation at 1.5 °C without light led to an efficient direct plantlet conversion of about 80%, the genotypes Oase and Chary × Krypton achieved no high amount of directly regenerated plantlets.

Tab. 14 Direct embryo to plant conversion rate of microspore derived embryos per genotype cultivated on four different shoot regeneration media (left) and five culture conditions (right). Depicted are means over four shoot regeneration media (left)/ five culture conditions (right) and three experiments. Additionally, overall means for the genotypes and means for the best combination of shoot regeneration media and culture condition for cultivation of microspore derived embryos are shown.

	Direct embryo to plant conversion rate [%]										
	Shoot regeneration medium (M)					Culture condition (C)					
Genotype (G)	**Mean**	**B5 +0.1mg/L GA_3**	**B5**	**B5 +0.1mg/L IBA +0.2mg/L BAP**	**MS**	**CR 26°C (12h)**	**LT 4°C (24h)**	**LT 4°C, (0h)**	**LT 1.5°C (8h)**	**LT 1.5°C, (0h)**	**Best media (B5+0.1mg/L GA3) x best culture condition (LT1.5°C, 0h)**
Krypton	78.7	83.3	75.2	73.5	71.7	20.8	92.7	86.5	89.6	90.0	95.2
Krypton × DSV2	76.3	84.2	76.1	75.1	70.0	20.8	91.7	89.6	89.6	90.0	100.0
Favorite × DSV2	68.7	75.7	69.4	67.2	62.5	30.2	71.0	63.5	93.5	85.3	91.7
DSV2	56.5	60.8	60.0	55.8	49.2	8.3	77.1	45.8	68.8	82.3	75.0
DSV1 × Adriana	68.3	75.8	70.0	62.5	65.0	57.3	62.5	66.7	76.0	79.2	87.5
Adriana ×Oase	66.7	71.2	66.7	70.8	57.5	29.2	76.0	74.0	73.0	81.3	91.7
DSV1	75.7	75.7	69.4	67.2	62.5	8.3	77.1	46.1	68.8	82.3	87.5
Express × Charly	56.5	60.8	60.0	55.8	49.4	8.3	77.1	46.1	68.8	82.3	87.5
Oase × NK Beauty	52.5	59.2	53.3	54.2	43.3	17.7	50.0	54.2	66.7	74.0	83.3
Komando × Express	41.3	50.8	44.3	33.9	36.3	12.5	47.7	49.4	41.4	55.6	66.7
Charly	32.9	37.5	37.5	29.2	27.5	24.0	34.4	35.4	32.3	38.5	33.3
Oase	32.1	35.0	35.0	33.3	25.0	11.5	34.4	38.5	37.5	38.5	33.3
Charly × Krypton	29.4	35.0	28.3	27.5	26.7	10.4	32.3	31.3	27.1	45.8	58.3
Mean	55.8	62.0	57.3	54.3	49.7	21.6	63.0	57.3	66.0	71.4	71.0
LSD5%G	4.2										
LSD5% C	2.6										
LSD5% M	2.8										
LSD5% G x C									9.3		
LSD5% G x M					8.3						

3.5 Discussion

In this study the effect of culture conditions, shoot regeneration media and of the genotype was examined on the direct conversion of microspore derived embryos to plantlets to improve the efficiency of the regeneration system.

3.5.1 Cold exposure of microspore derived embryos

High temperature of 32 °C for two days is routinely used in microspore culture to induce their sporophytic development. Gu et al. (2004) showed that a pretreatment (4°C) of flower buds subjected to a liquid medium at low temperature (4°C) for two and four days could also enhance microspore embryogenesis. Low temperature as stress induction can also be applied to induce direct conversion of microspore derived embryos to plantlets without undergoing secondary embryogenesis. Previous studies (Kott and Beversdorf 1990, Fletcher et al. 1998, Zhang et al. 2006) reported a positive effect of exposing microspore derived embryos to 4 °C and a 16-hour light/day photoperiod for 14 days on their direct embryo to plant conversion. Cegielska-Taras et al. (2002) studied the influence of low temperatures (1 °C, 4 °C) linked with a short photoperiod (eight hours light/day) and high temperature (24 °C) linked with long photoperiod (16 hours light/day) on microspore derived embryos conversion. The two-week cold treatment at 1 °C led to an over 70% direct embryo to plant conversion rate, compared to around 20% at 4 °C.

Our study presented here attempts to evaluate the effect of different cold treatments to find an appreciate application for a wide spectrum of genotypes of *Brassica napus*. The choice of these variants is caused by previous experiments (data were not shown). In the first experiment of this study, we cultivated microspore derived embryos of five genotypes for 14 days at 4 °C linked with continuous and eight hours light and under standard conditions. The scored direct embryo to plant conversion rate was two times higher after cold treatment with continuous light (28%) compared to cultivation under standard conditions (14%). The cold treatment linked with eight hours light led also to a significant increase of direct embryo to plant conversion rate (26%). The multiplication of the shoots was enhanced by this variant for the tested genotypes. Therefore in the following second experiment this variant was examined for 13 genotypes.

Here, the microspore derived embryos were cultivated at 4 °C under continuous light and darkness and at 1.5 °C under eight hours light and continuous darkness. The best mean over

13 genotypes for direct embryo to plant conversion was observed with microspore derived embryos cultivated at 1.5 °C under continuous darkness (71.4%). In contrast to the first experiment including five genotypes, the 14-day cold treatment at 4 °C with and without continuous light resulted for 13 tested genotypes in nearly 60% direct embryo to plant conversion frequency. The different tested cold treatments were not significantly different (LSD5% C=2.6 see Tab. 14). The results of this work showed that cold treatment at 4 °C and at 1.5 °C with different photoperiod (24 h, 8 h) and without light significantly increased the percentage of directly regenerated plantlets.

For induction of direct conversion of microspore derived embryos to plantlets the photoperiod was not necessary and could not significantly improve the development of microspore derived embryos to plantlets. In general, in angiosperms, the length of photoperiod determines the shift from vegetative to generative development. While a photoperiod under 12 hours light/day causes vegetative growth, a 16-hour light/day photoperiod promotes the generative growth, e.g. buds formation and flowering which is controlled by the FT-gene (Rapacz 2002). For vernalisation of winter rapeseed an eight-week cold induction at low temperature and a photoperiod of eight hours light a day is applied (Van Deynze 1993).

In contrast to the photoperiod, low temperature plays an essential role in direct conversion of microspore derived embryos to plantlets. During cold treatment of microspore derived embryos under 8 or 16 hour light we observed no or no remarkable growth. Obviously, the growth of the embryos is inhibited because in general, next to light intensity and spectrum the efficiency of photosynthesis depends on temperature. Additionally, low temperature causes a clearly reduced efficiency of photosynthesis. Under continuous darkness no photosynthesis occurred. Best frequencies of 100% were obtained at 1.5 °C without light (see Tab. 14).

Kott and Berversodorf (1990) suggested that the microspore derived embryo is in a kind of “chilling modus” comparable to a zygotic embryo in a dormant seed. They assumed that the morphology and physiology of microspore derived embryos and zygotic embryos are largely similar, although they have different origins and developmental environments. By comparison with a dormant, non-germinating seed embryo, they further suggested that by cold treatment the high endogenous abscisic acid level inhibiting the germination in microspore derived embryos is reduced and promote this process. Takeno et al. (1983)

showed in somatic grape that three to five weeks of chilling and treatments led to embryo germination. Wareing and Saunders (1975) constructed the hormone-balance-theory by explaining seed dormancy by the more or less simultaneous operation hormones promoting (GA_3) and inhibiting (ABA) germination: Increasing GA_3 levels inhibits the ABA-level and lead to seed germination by breaking the dormancy. Current studies (Ali-Rachedi 2004, Schatzki et al. 2013) discuss the complex mechanism of breaking the seed dormancy and further research is still necessary for understanding.

Zhang et al. (2006) published that an efficient direct embryo to plant conversion could be achieved by treating microspore derived embryos with certain stress conditions such as cold treatment, partial desiccation after ripening or cotyledon excision (at late cotyledonary stage). Without their cotyledons, nutrition in the embryos could concentrate on the process of direct conversion to plantlets (Xu et al. 2007). Kott and Beversdorf (1990) suggested that a drying treatment of microspore derived embryos initiate the conversion process of microspore derived embryos.

In the study of Zhang et al. (2006), a one-day desiccation treatment of microspore derived embryos on filter paper in Petri dishes under a 16 hours light/day photoperiod increased the frequency of directly regenerated plantlets for all tested genotypes. A combination of different stress conditions, i.e. one-day desiccation followed by a two-week cold treatment and cotyledon excision may enhance the positive effect on direct plantlet regeneration. For a short one-day desiccation of cotyledonary-staged embryos and cold treatment, microspore derived embryos could be transferred onto filter paper in Petri dishes without solidified media. The cold induction of microspore derived embryos on solidified media seems not to be necessary because the microspore derived embryos seem to rest and the cotyledons contain essential nutrients. After the two-week cold inductions may follow the cotyledon excision and the transfer of microspore derived embryos on efficient shoot regeneration media.

3.5.2 Microspore derived embryos cultivation on shoot regeneration media with and without phytohormones GA_3, BAP and IBA

In literature, microspore derived embryos at cotyledonary stage were transferred to MS media (Lighter 1989, Yeung 1996) or B5 media (Baillie et al. 1992). Zhou et al. (2002) reported that microspore derived embryos were cultivated on MS media for shoot

development and after four weeks of cultivation they were transferred to MS media supplemented with phytohormones (BAP, IAA) for root development (successive subculture).

In this study, we examined the direct plantlet development of microspore derived embryos cultivated on MS and B5 medium. By comparison of B5 and MS medium, B5 medium led to a higher percentage of directly regenerated plantlets. We suggested that the nutrient composition of B5 medium promote the direct conversion of microspore derived embryos to plantlets. In contrast to MS medium developed by Murashige and Skoog (1962), the levels of inorganic nutrients in the B5 medium by Gamborg et al. (1968) are lower. Some nutrients are not contained in B5 medium (ammonium nitrate and sulphate, sodium dihydrogen phosphate, potassium phosphate, glycine). The concentration of the B1-vitamine thymine is 10 mg/L instead of 0.1-1 mg/L. The basal medium was modified by Gamborg et al. (1968) for cell cultures of soy bean. Its nutrient composition seems to stimulate shoot development of microspore derived embryos.

Further, in this study the effect of the phytohormones gibberellic acid ($GA_{3)}$, 6-benzylaminopurine (BAP) and indole-3-butyric-acid (IBA) on direct embryo to plant conversion was analysed. The phytohormone GA_3 is generally used in plant tissue to stimulate stem elongation. In current studies (Corredoira et al. 2003, Junaid et al. 2006 and 2007) it was reported, that GA_3 induced enhanced somatic embryo germination in *Castanea sativa* and *Catharanthus roseus*. In addition, short application of GA_3 was found by Junaid et al. (2007) to be very effective in maturation before conversion. Hays et al. (2001) examined the role of gibberellins during early embryo development of *Brassica napus*. An inhibitor of GA-biosynthesis, uniconazole, was applied at the globular stage of development (ten days after microspore isolation) when endogenous GA_1 levels are increasing rapidly. To the early torpedo stage no apparent effect of the inhibitor was observed. By 25 days after microspore isolation uniconazole-treated microspore derived embryos showed significantly reduced axis elongation (50%). In some studies (Cosgrove and Sovonick-Dunford 1989, Yang et al. 1996, Cowling and Harberd 1999) cell elongation has been reported to play an important role in GA-regulated stem elongation. Furthermore, this phytohomone operate as potential antagonist of absisic acid in process of seed stimulation (Wareing and Saunders 1971, Ali-Rachedi 2004). In general, the gibberellic acid mechanism is still inadequately understood. Cegielska-Taras et al. (2002) analysed the effect of gibberellic acid in a concentration of 0.1

and 1 mg/L on direct embryo to plant conversion but observed no significant effect of the phytohormone gibberellic acid on the stimulation of shoot development from the apical meristem of microspore derived embryos in *Brassica napus* L. ssp. *oleifera*. Nevertheless, they postulated a slightly higher number of embryos with direct shoot formation in each temperature variant in media containing 0.1 mg/L GA_3. In Brassica napus L., Haddadi et al. (2008) reported that 0.1 mg/L GA_3 improves plantlet regeneration via elongation of the embryo axis and also by accelerating embryo maturation. Ahmadi et al. (2012) examined different GA_3 concentrations (0.05-0.15 mg/L) and obtained with 0.05 and 0.1 mg/L GA_3 the highest percentage of normal plantlet regeneration (40%). In this study, considering all experiments (Fig. 1 -2), the best direct embryo to plant conversion rate an average was achieved by the use of for B5 with 0.1 mg/L GA_3. Microspore derived embryos cultivated on B5 media with 0.1 mg/L GA_3 showed improved direct embryo to plant conversion, regardless of the cold treatment and genotype (except Krypton, see Tab. 2.2). In contrast to the first experiment, the interaction between genotype and shoot regeneration media was not significant. Both experiments showed a highly significant interaction of the shoot regeneration media and the culture condition.

In addition to GA_3, the effect of the phytohormones 6-benzylaminopurine (BAP) and indole-3-butyric-acid (IBA) on direct embryo to plant conversion was examined. BAP is generally used in plant tissue culture for shoot development and multiplication (Tang et al. 2003). Zhang et al. (2006) reported a positive effect of BAP on microspore derived embryos to shoot development revealing the concentration of 2 mg/L BAP to be the most convenient.

The phytohormone IBA, belonging to the auxin-family, is known to stimulate extension growth and root building and is deemed to be a precursor of indole-3-acetic acid (IAA). IAA is the most abundant auxin natively occurring and functioning in plants. It generates the majority of auxin effects in intact plants and is the most potent native auxin. Ahmadi et al. (2012) reported that the combination of 0.1 mg/L BAP and 0.2 mg/L IBA resulted in the highest percentage of normal plantlet regeneration in *Brassica napus* L. (37%). In this study, BAP concentrations of 0.1 mg/L and 0.2 mg/L in combination with 0.1 mg/L GA_3 and 0.1 mg/L IBA were tested. Regarding all experiments, microspore derived embryos cultivated on B5 with 0.1 mg/L GA_3 and 0.1 mg/L IBA and 0.2 mg/L BAP obtained high rates for direct embryo to plant conversion.

3.5.3 Genotype dependence of direct embryo to plant conversion of microspore derived embryos

A number of studies reported that direct embryo to plant conversion mainly depends on the genotype (Chuong et al. 1988, Kontowski and Friedt 1994). In agreement to literature, results of this work showed large differences between tested genotypes regarding their ability to convert directly to plantlets (Tab. 12 and Tab. 14).
Surprisingly, the second experiment including 13 genotypes showed that the efficiency of microspore derived embryos directly converting to plantlets depended on the choice of culture condition which was more important than the choice of the genotype. Nevertheless the genotype of the donor plants is still an important factor and mainly influences the efficiency of microspore culture and direct embryo to plant conversion.

3.6 Conclusion

Microspore derived embryos cultivated on B5 media with 0.1 mg/L GA_3 and exposed to a cold treatment at 1.5 °C for a period of 14 days in darkness showed a highly improved direct embryo to plant conversion. Following this, laborious and time consuming sub-culture stages for direct shoot regeneration could be significantly reduced. The present method to improve conversion of microspore derived embryos directly to plantlets could prove to become a standard procedure in plant breeding programs for cultivation of doubled-haploid lines. Our study postulated the advantage of cold-treated microspore derived embryos compared to microspore derived embryos cultivated under standard conditions (26 °C, 12 h light).

3.8 Acknowledgments

The technical assistance of Rosemarie Clemens is greatly appreciated. The authors gratefully acknowledge the Federal Ministry of Economy & Technology and the Gemeinschaft zur Förderung der Privaten deutschen Pflanzenzüchtung (GFP e.V.) for funding this research project (ÖE 134/09 NR).

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Chapter 4:

Final Discussion

Importance of light intensity, light quality and temperature in production of doubled haploid lines

The influence of donor plant growth conditions on microspore embryogenesis has been the object of research in several previously conducted studies. For example, significantly higher embryo yields were obtained from microspore culture initiated from donor plant grown at 10/5 °C (Ferrie and Keller 1995, Ferrie and Caswell 2011) linked with 16 hours light/day. In these studies, measured light intensities in growth chambers were indicated from 200 to 400 µmol m^2 s^{-1} and were confirmed by our conducted measurements (Li-1400 Data Logger, Licor Biosciences, Nebraska, Canada). Sodium-vapour lamps (SON T Agro Philips Master Green Power CGT 400 watt**,** Philips, Germany) with a red-light fraction were applied behind glass with 1 m distance to the donor plants. Additionally, fluorescent tubes (Osram Flora 77= 2*36 watt and 2*58 watt, München, Germany) were installed to promote photosynthesis performance of the plants because the process of photoperiodism is mainly influenced by the wavelength of the light. Beneficial for photosynthetic activity and well-developed plants is a wavelength between 400 and 700 nm with a balanced spectrum. For example, the far red fraction of the spectrum induced the phytochrome reaction which determines whether the plant remains in the vegetative stadium or will undergo the generative development while a deficit of the blue spectrum can cause excessive stem growth in plants (http://www.dhlicht.de/download/pflanzenbroschuere.pdf).

The microspore culture incubation in *Brassica* requires high temperatures. Therefore microspore culture including an antimitotic agent was incubated at 32 °C for 36 hours in darkness following by 7-day incubation at 28 °C. The high temperature is considered to be a pre-requisite for the switch from the gametophytic to the sporophytic development of microspores (Custers et al. 1994, Prem et al. 2012).

Prem et al. (2012) developed an *in vitro* system at lower temperature (18 °C) to efficiently induce microspore embryogenesis through different pathways. They suggested that low temperature applied for longer periods preferably lead to an embryogenesis pathway whose

first division originates asymmetric cell identities, early polarity establishment and the formation of suspensor-like structures, mimicking zygotic embryogenesis. This new *in vitro* system could be a model system which could be helpful to analyse the mechanisms of microspore development to microspore derived embryos *in situ*.

After incubation the microspore cultures were transferred on a rotary shaker in the culture room at 26 °C and 12 hours light with consequential chlorophyll activation. A fluorescent tube was applied in a distance of 50 cm to the plates. The light intensity amounted to 80 µmol m^2 s^{-1}.

For cultivation of microspore derived embryos in culture room conditions fluorescent tubes (Osram Cool white type: Lumi Lux daylight, München, Germany) were used with measured light intensities of 70 µmol m^2 s^{-1} (shelf ground). The choice of growth factors (temperature, light intensity, photoperiodism, and relative humidity) which are plant species- specific were realised for efficient *in vitro* plantlet development in oilseed rape.

For cold induction (Chapter 2) in Light Thermostat also fluorescent tubes were used. The temperature was reduced to 1.5 °C and 4 °C. The light intensity was 100 µmol m^2 s^{-1}. After a two-week cold induction at low temperatures the microspore derived embryos showed no growth by comparison with microspore derived embryos cultivated in the culture room. Next to light intensity and spectrum, the efficiency of photosynthesis depends on temperature, for this reason we suggested that under continuous light at 1.5 °C and 4 °C the photosynthesis was mainly reduced and consequently, under continuous darkness no photosynthesis occurred. For further research it is necessary to measure the output of “embryo oxygenation”. The higher the oxygen output, the higher the photosynthesis frequency. We hypothesized that the embryo rests like a dormant zygotic embryo in a seed.

Directly regenerated plantlets were transferred to soil, covered with a plant bonnet and exposed to a growth chamber at 15 °C for 16 hours day length and a low light intensity of 40 µmol m^2 s^{-1}. Applying low light intensities seems to be important for an adaptation from *in vitro* to *ex vitro* before transferring to the greenhouse (Zhang et al. 2003).

Embryo age

Embryo age was scored from the day of microspore isolation to the day of transfer to solid media. Regarding all experiments (Chapter 2 and 3), the embryo age widely ranged from 12 to 42 days old embryos because in our studies the microspore derived embryos were

selected by their size (see Embryo size). Kott and Beversdorf (1990) observed in *Brassica napus* L. ssp. *oleifera* three to five fold greater germination rates for 35 days old embryos compared to embryos being only 21 days old. In general, the germination rate was declined for embryos being between 35 and 49 days old. Kott and Beversdorf (1990) defined the germination rate "as the point in time when a somatic embryo begins to develop a shoot and a root". For this study the direct embryo to plant conversion rate was scored when microspore derived embryos directly developed shoots without secondary embryogenesis. The root growing was not scored because the microspore derived embryos were not transferred to soil and grown to plants. In general, the embryo age seems to influence the direct embryo to plant conversion of microspore derived embryos. In our study, most of the used embryos were not older than 20 to 23 days; per chance the scored conversion rate could be increased by analysing embryos at an age of 35 days. Indeed, the approach was to improve the direct embryo to plant conversion in a way which is feasible and time/cost-saving for a plant breeding company. Using 35 day old embryos would lead to an increased cultivation period of the microspore derived embryos from 12 to 15 days. However, it is questionable if this proves to be sustainable because including these 12 to 15 days and the two-week cold treatment, the cultivation time for microspore derived embryos is extended about four weeks compared to standard cultivation. On the contrary, up to now most of the microspore derived embryos under standard conditions need to be sub-cultivated repeatedly for direct embryo to plant conversion, due to secondary embryogenesis, which is also time/and cost-consuming. Furthermore, Kott and Beversdorf (1990) reported in *Brassica napus* a higher germination rate of 28 days old embryos than of 21 day old embryos, regardless of the exposed temperature (at 0 °C, 2 °C, 4 °C, 25 °C). Additionally, they observed for cultures that responded poorly at 25 °C, a high increase in germination after a ten-day cold period, regardless of the embryo age.

In this study (Chapter 3) the direct embryo to plant conversion of microspore derived embryos could be improved after a 14-day cold treatment for an embryo age between 20 to 27 days for all used genotypes. Microspore derived embryos of all genotypes responded poorly under standard conditions (26 °C). After the cold exposure the direct embryo to plant conversion rate dramatically increased. Results of Cegielska-Taras et al. (2002) in *Brassica napus* L. ssp. *oleifera* could also be confirmed; whereas in their study, they used 21-day old embryos and a 14-day cold treatment at 4 °C and 1 °C.

Embryo size

In cultures with a high density of embryos (500-800 per petri dish), the embryos were separated in a few Petri dishes for rapid growing. All embryos used for the experiments had nearly the same size (0.7 cm to 1.0 cm). Cultures including less than 50 embryos were discarded because the microspore derived embryos were rapidly growing and enormously large (over 1 cm). In contrast, in cultures with a very high embryogenesis over 1000 embryos per Petri dish, microspore derived embryos were slowly growing and most of the latest embryos showed an abnormal development. For the experiments, only well-developed embryos were used. Zhang et al. (2006) examined the effect of embryo length. In their study the highest rate of plant development was obtained with an embryo size of 2 mm to 4 mm and embryos with a size about 4 mm resulted in a decrease of plant development. Ahmadi et al. (2012) confirmed that embryo size of 2 mm to 4 mm resulted in highest direct plantlet development. The effect of embryo size on direct embryo to plant conversion and diploidisation of microspore derived embryos was analysed in further experiments (data not shown). The embryos were sorted in large (7-10 mm), medium (5-7 mm) and small (<5 mm). 17 genotypes including cultivars and their F1-crosses were studied. The analysis of variance showed no significant effect of embryo size on the traits direct embryo to plant conversion and diploidisation of microspore derived embryos. The variance components indicated a predominant effect of the genotype on the traits and showed highly significant effects of genotype and embryo size interaction. Regarding all genotypes, the embryo group large and medium resulted in 64% diploid plantlets. The group small achieved a diploidisation frequency of 66%. Large- and medium-sized embryos led to fewer diploid plantlets. Medium-sized embryos obtained more directly regenerated plantlets (16%) than small and large embryos (14%).

Furthermore, we recently studied the effect of different embryo sizes under cold treatment; embryos with a size less than 7 mm often died (data not shown). For that reason, we only used embryos with a size of at least 7 mm.

Alternatives to cold induction for improvement of direct embryo to plant conversion frequency

One of the objectives of this work was the improvement of direct embryo to plant conversion of microspore derived embryos. Besides the exposing of the microspore derived embryos to low temperatures, many previous experiments were conducted to avoid secondary embryogenesis. One experiment was the desiccation or air drying of microspore derived embryos under the clean bench. The embryos were desiccated from 0.3 up to 1.5 hours. Most of the embryos died. The direct embryo to plant conversion rate was scored after four weeks of cultivation on culture media and resulted in 8.3% to 27.8 % dependent on the tested genotype. The results indicated that rapid air drying under clean bench for 1 hour led to the highest frequencies but simultaneously also to a high percentage of dead embryos (from 16% to 50%). In some cases, desiccated embryos developed degenerated shoots and showed no normal plantlet regeneration. Using a desiccation method by rapid air drying under clean bench, Kott and Beversdorf (1990) observed highest germination frequencies of up to 60% with a 0.5-hour and 1-hour treatment and led to 14% dead embryos. For this method embryos were exposed to the air stream of laminar flow bench in plates with liquid medium without lids. The plates were inclined for draining away from the embryos. For optimum air condition the embryos were separated from each other as much as possible. By slow air desiccation for 3 to 24 days they reported of germination frequencies with a range from 46% to 100% compared to the control with 27%. For this method the microspore derived embryos were transferred on dry filter paper in a Petri dish layered with parafilm and cultivated at 25 °C in culture room. A combination of different methods, for example with cold treatments (see Chapter 3, this work) may improve the direct embryo to plant conversion rate, for recalcitrant genotypes, too.

Development of a "Dry Artificial Seed System" for long-term storage of microspore derived embryos

In plant breeding programs storage of microspore derived embryos could be beneficial for synchronisation of doubled haploid production for direct transfer to the field in August or September. Because of limited greenhouse capacities only a reduced number of plants could be provided for the field transfer. Up to now, there is no sufficient, existing method for long-term storage of microspore derived embryos. Already realised in practice is the storage of

microspore derived embryos in liquid induction media up to three month at low temperature without light. We conducted several experiments to analyse the ability of long term storage of microspore derived embryos. The cold storage of more than three month resulted in yellowish dead embryos, although the media was changed after three month of storage.

Furthermore, the cold storage of microspore derived embryos at 4 °C in the Light Thermostat under continuous light and eight hours light on solid media in plastic boxes was also tested. After eight weeks of incubation most of the embryos were dead. A lot of condensed water was accumulated on the solid media and consequently the rate of contamination (Bacteria diseases) increased with extended storage. However a storage period of about eight weeks was tested but could not be used as an alternative to simultaneously induce vernalisation of the microspore derived embryos.

Furthermore, we conducted an experiment following a desiccation protocol developed by Senaratna et al. (1991) and optimised for microspore derived embryos of broccoli by Takahata et al. (1993). According to that, microspore derived embryos at different developmental stages (from 4 mm up to 1.5 cm) were treated with 1 x 10^{-4} M abscisic acid for 24 hours and were desiccated slowly over a six day period on filter paper in a Petri dish. The microspore derived embryos were exposed daily, in series, from higher relative humidity (RH) to lower RH desiccators in which the RH was kept constant by use of saturate solutions. The embryos were stored for four month at room temperature. After three month the microspore derived embryos were transferred to solid media but only one embryo survived. Takahata et al. (1993) reported about the highest desiccation tolerance of embryos after treatment with 1 x 10^{-4} M abscisic acid for 24 hours; on average 27% to 48% of the desiccated embryos converted to plants.

In a further experiment, the microspore derived embryos were transferred to liquid nitrogen for long-term storage following a protocol by Matsumoto et al. (1994). Before freezing, microspore derived embryos at different developmental stages were treated with a special dehydration solution PV2 (30% w/v Glycerol; 15% w/v Ethylene glycol, 15% w/v DMSO and 0.4 M Saccharose) or alternatively with only half the concentrations of PV2. After freezing in liquid nitrogen for 20 minutes, microspore derived embryos were slowly thawn in a water bath of 40 °C. After thawing microspore derived embryos were transferred first to 0.5 molar liquid MS media for 15 minutes and then to 1.2 molar MS media for 20 minutes. After

cultivation of microspore derived embryos on solid media for a period of seven days, the vitality was scored. In all experiments no embryo survived.

On the basis of the great amount of influencing factors and the disappointing results from the described experiment it is obvious that further investigations are necessary to develop a "Dry artificial seed system for microspore derived embryos" of *Brassica napus* L.

Conclusion

Although microspore culture in *Brassica napus* L. is already used as a routine technique, the overall utilisation in breeding programs is considered to be only intermediate due to its high laboratory costs and its efficiency needs to be further improved. The use of two Colchicine replacing, alternative antimitotic agents did not lead to significant higher diploidisation rates. Nevertheless the Colchicine induced diploidisation rate could be significantly improved but is still insufficient referring to a desired diploidisation frequency of about 90%. For that reason it might be of utmost importance to further identify new, putative antimitotic agents to additionally increase the diploidisation rates in *Brassica*.

Furthermore, in this study the direct embryo to plant conversion rate of microspore derived embryos could be significantly improved for all tested genotypes. Moreover, a combination of different stresses, e.g. one-day desiccation by slow air drying followed by a two-week cold treatment and cotyledon excision may avoid the secondary embryogenesis and could prove beneficial in the development of microspore derived embryos to doubled haploid plants.

For a better understanding of microspore culture mechanisms it might be highly necessary to expand research onto the genetic level because there are still unknown issues about the inheritance of traits like the microspore's capability to divide fast, to form diploid embryos efficiently or of the microspore derived embryos to convert directly to plantlets. The development of trait specific marker would promote an *in vitro* selection of the traits "the capacity of microspores to form spontaneously or antimitotic induced diploid embryos" and "the capacity of microspore derived embryos for direct shoot conversion" but also require a subsequent evaluation of putative genotypes under field conditions.

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Summary

Methodological improvements in microspore culture of *Brassica napus* L.

The utilisation of microspore *in vitro* culture and chromosome doubling for production of homozygous and doubled haploid plants is an important issue in modern breeding programs. Nevertheless, recalcitrant genotypes, secondary embryogenesis of microspore derived embryos and insufficient Colchicine induced diploidisation frequencies still represent major drawbacks for an efficient application of the doubled haploid technology. For that reason the following work, consisting of two studies, was conducted to further improve the doubled haploid technology in oilseed rape (*Brassica napus* L.).

The aim of the first study was to enhance the Colchicine induced diploidisation frequency and to test alternative, less toxic antimitotic agents with similar performance. Furthermore, this study was conducted to investigate the effect on the direct embryo to plant conversion of microspore derived embryos. In contrast to the whole plant Colchicine treatment, the direct application of Colchicine to isolated microspores reduces the risk potential and proved to enhance the diploidisation frequency in doubled haploid production. But although the direct application reduces the amount of Colchicine, concentrations are still high and therefore require, due to its toxicity, an appropriate waste storage and an expensive disposal. Promising, less toxic alternatives with a high affinity to plant tubulin are the herbicides Amiprophos-methyl (APM) and Pronamide which could be applied to microspore culture in low concentrations.

In contrast to previous studies, the antimitotic agents APM, Pronamide and Colchicine were examined in different concentrations for 72 hours, alone and in combination to detect putative synergistic effects on the diploidisation frequency. The influence of the antimitotic treatments on the ability of microspore derived embryos to convert directly to plantlets was also studied. The results showed no synergistic effect of APM, Pronamide and Colchicine in combination on diploidisation frequency of eight different winter oilseed rape genotypes. The tested genotypes resulted in 40% to 64% diploid plantlets (means from all treatments). The diploidisation frequency of the treatments varied from 33% (3 µM APM) to 70% (25 µM Colchicine) and the direct embryo to plant conversion ranged from 14% to 23%.

In a second experiment, the effect of different Colchicine concentrations (250, 150, 125, 25 µM) on diploidisation and direct embryo to plant conversion frequency was analysed. In

general, the duration time of incubation was 72 hours, but the treatment with 250 µm Colchicine was additionally incubated for 48 hours. The four tested genotypes showed a diploidisation rate from 58% to 66%. The highest mean of 77% doubled haploid plantlets was achieved by the treatment with 250 µM Colchicine incubated for 48 hours. The direct embryo to plant conversion rate varied from 9% to 23%.
In a further experiment, the effect of a low and a relatively high dimethyl sulphoxide (DMSO) concentration in combination with a standard Colchicine (250µM, 36h) treatment was studied on diploidisation and direct embryo to plant conversion rate of four winter oilseed rape genotypes. This experiment was conducted because in practice antimitotic agents are usually dissolved in DMSO with a final concentration from 0.03% to 3%. No significant effect of both applied DMSO-concentrations on diploidisation- and direct conversion frequencies of microspore-derived embryos to plantlets could be detected.
In addition, the spontaneous and Colchicine induced diploidisation frequencies of 17 winter oilseed rape genotypes including cultivars and their F1-crosses were examined and the ability of their microspore derived embryos to convert directly to plantlets was evaluated. The spontaneous induced diploidisation frequency showed a wide range from 15% to 69% and the colchicine induced diploidisation frequency ranged from 40% to 83%. For all tested genotypes, the direct embryo to plant conversion rate widely varied from 2% to 35%.
Regarding all experiments of the first study, a significant influence of the antimitotic treatments (Colchicine, APM, Pronamide) on the ability of microspore derived embryos directly converting to plantlets could not be detected. The observed spontaneous and antimitotic induced diploidisation and the direct embryo to plant conversion frequency were genotype dependent. Further investigations are necessary to obtain an efficient diploidisation frequency of about 90%.

Usually, most of the embryos undergo secondary embryogenesis which requires laborious, time- and cost-intensive multiple sub-culturing of the shoots. Because of that the aim of the second study was the improvement of the direct embryo to plant conversion frequency of microspore derived embryos and the reduction of secondary embryogenesis.
In the first experiment the effect of ten shoot regeneration media supplemented with and without phytohormones (gibberellic acid, indole-3-butyric acid and 6-benzylaminopurine) and a 14-day cold treatment at 4 °C (Light Thermostat) of microspore derived embryos on

direct embryo to plant conversion frequency was tested. The cold treatment with 4 °C was linked with eight hours and continuous light. For standard cultivation microspore derived embryos were exposed to 26 °C and 12 hours light. The five tested winter oilseed rape cultivars showed a range from 13% to 39% directly converted plantlets. The cultivation of microspore derived embryos on Gamborg B5 media supplemented with 0.1 mg/L gibberellic acid achieved the highest mean of 43%, while cultivation on B5 media supplemented with 0.1 mg/L indole-3-butyric acid and 0.2 mg/l 6-benzylaminopurine resulted in a mean of 11%, only. The two-week cold treatment significantly increased the frequency of direct embryo to plant conversion from 14% (under standard conditions) to 28%.

In a second experiment, the effect of the four, previously most efficient, media and a 14-day cold treatment at 1.5 °C and at 4 °C (Light Thermostat) were tested on direct embryo to plant conversion frequency. The cold treatment at 1.5 °C was linked with eight hours of light and also with continuous darkness, while the cold treatment at 4 °C was linked with continuous light and continuous darkness. This experiment was conducted with 13 winter oilseed rape genotypes including cultivars and F1-hybrids and showed a wide range from 29% to 76% of direct embryo to plant conversion rate. In comparison to cultivation under standard conditions, the cold treatment at 1.5 °C and at 4 °C with and without light significantly increased the number of directly regenerated plantlets from 21% to 71%. For the tested culture media, the variation of direct embryo to plant conversion frequencies ranged from 50% (Murashige and Skoog) to 60% (Gamborg B5 with 0.1 mg/L gibberellic acid). Two of 13 genotypes resulted in 100% directly regenerated plantlets by cultivation of microspore derived embryos on B5 with 0.1 mg/l gibberellic acid and a 14-days cold induction at 1.5 °C without light.

Results of the second study showed that although the ability of microspore derived embryos to convert directly to plantlets is mainly genotype dependent, a 14-days cold induction at 1.5 °C and at 4 °C significantly increased the direct embryo to plant conversion frequency. In contrast to that the choice between the most efficient culture media (MS, B5, B5 with 0.1 mg/L gibberellic acid and B5 with 0.1 mg/L indole-3-butyric acid and 0.2 mg/L 6-benzylaminopurine) turned out to be only a subordinated factor for the improvement of the direct embryo to plant conversion frequency. In conclusion, an efficient direct embryo to plant conversion of about 70% could be achieved for most of the tested genotypes by cold treatment at 1.5 °C without light.

Zusammenfassung

Methodische Verbesserungen der Mikrosporenkultur in *Brassica napus* L.

Bei der routinemäßigen Anwendung der Mikrosporenkultur zur Herstellung doppelt-haploider Linien kommt es bis heute zu Engpässen in der praktischen Raps-Züchtung. Die Hauptprobleme stellen eine unzureichende Colchizin-induzierte Diploidisierungsrate und eine niedrige direkte Regeneration von Pflanzen aus Mikrosporen-Embryonen dar.

Die vorliegende Doktorarbeit besteht aus zwei Studien, die sich mit dem Thema: „Methodische Verbesserungen der Mikrosporenkultur in *Brassica napus* L.“ auseinandersetzen.

Ziel der ersten Studie war die Erhöhung der Colchizin-induzierten Diploidisierungsrate von Mikrosporen. Außerdem wurde aufgrund der hohen Toxizität von Colchizin nach alternativen, weniger toxischen Mitosehemmstoffen gesucht, die einen hohen Diploidisierungserfolg ermöglichen, ohne die direkte Konversion der Mikrosporen-Embryonen zu Regeneraten mit direkten Sprossansätzen zu verringern und damit die Entwicklung zu doppelt-haploiden Pflanzen zu verzögern. Im Vergleich zur Colchizinierung ganzer Pflanzen, minimiert die Behandlung isolierter Mikrosporen zwar das Gefährdungspotential für Mensch und Umwelt und erzielt um ein Vielfaches höhere Diploidisierungsraten, dennoch ist eine Anwendung von Colchizin mit einer angemessenen Gefahrstofflagerung und Entsorgung sowie mit hohen Kosten verbunden. Zudem wird Colchizin im Vergleich zu anderen Mitosehemmstoffen immer noch in relativ hohen Konzentrationen appliziert.

Als weniger toxische Alternativen bieten sich die Herbizide APM und Pronamid an, die beide eine hohe Affinität zu Pflanzentubulin besitzen und daher in geringeren Konzentrationen direkt in der Mikrosporenkultur appliziert werden können. Im Gegensatz zu bereits vorhanden Studien wurden die drei Mitosehemmstoffe APM, Pronamid und Colchizin in unterschiedlichen Konzentrationen allein und in Kombination appliziert, um einen potentiellen synergistischen Effekt auf die Diploidisierungsrate detektieren zu können. Daneben wurde der Einfluss der Mitosehemmstoff-Behandlungen auf die Rate der direkten Konversion der Mikrosporen-Embryonen zu Regeneraten mit direkten Sprossansätzen untersucht. Eine Kombination der Mitosehemmstoffe APM, Pronamid und Colchizin führte zu keiner effizienten Diploidisierungsrate; ein synergistischer Effekt der Mitosehemmstoffe

konnte nicht nachgewiesen werden. Die acht getesteten Winterrapsgenotypen erzielten eine Diploidisierungsrate von 40% bis 64%. Die Mitosehemmstoff-Behandlungen der isolierten Mikrosporen variierten zwischen 33% (3 µM APM, 72 Stunden) und 70% (25 µm Colchizin, 72 Stunden). Nur 14% bis 23% der Mikrosporen-Embryonen konvertierten direkt.

In einem zweiten Versuch wurde der Effekt unterschiedlicher Colchizinkonzentrationen (250, 150, 125, 25 µM) auf die Diploidisierungsrate und die direkte Konversionsrate der Mikrosporen-Embryonen untersucht. Die Inkubation erfolgte für 72 Stunden. Die Mikrosporen wurden für die Behandlung mit 250 µM Colchizin außerdem für 48 Stunden inkubiert. Die vier getesteten Genotypen zeigten eine Colchizin-induzierte Diploidisierungsrate von 58% bis zu 66%. Die höchste mittlere Diploidisierungsrate wurde mit der Behandlung 250 µM Colchizin und einer Behandlungsdauer von 48 Stunden erzielt. Die direkte Konversionsrate der Mikrosporen-Embryonen zu Regeneraten mit direkten Sprossansätzen variierte hingegen von 9% bis 23%.

In einem weiteren Experiment wurde der Einfluss einer niedrigen und einer relativ hohen Konzentration an DMSO in Kombination mit einer Colchizin-Behandllung (250 µM, 72 Stunden) auf die Diploidisierungs- und die direkte Konversionsrate der Mikrosporen-Embryonen untersucht. Hierbei wurden Mikrosporen-Embryonen von vier Winterrapsgenotypen verwendet. Hintergrund für die Untersuchung von DMSO ist dessen häufiger praktischer Einsatz als Lösungsmittel für Mitosehemmstoffe, wobei die hier verwendeten Konzentrationen von 0.03% und 3% an in der Praxis gebräuchliche Mengen angelehnt wurden. Es konnte jedoch kein signifikanter Einfluss dieser beiden applizierten DMSO-Konzentrationen auf die Diploidisierungs- und die direkte Konversionsrate der Mikrosporen-Embryonen zu Regeneraten mit direkten Sprossansätzen beobachtet werden.

In einem weiteren Versuch wurden 17 Winterrapsgenotypen bezüglich ihrer spontanen und ihrer Colchizin-induzieren Diploidisierungsrate analysiert. Desweiteren wurde die direkte Konversionsrate der Mikrosporen-Embryonen bestimmt. Die ausgewählten Genotypen enthielten sowohl Sorten als F1–Hybriden. Die spontan-induzierte Diploidisierungsrate zeigte eine große Variation von 15% bis 69%. Im Vergleich dazu, erreichte die Colchizin-induzierte Diploidisierungsrate Werte von 40% bis 83%. Die Mikrosporen-Embryonen der getesteten Genotypen wiesen ebenfalls eine große Spannbreite bezüglich ihrer direkten Konversationsrate auf. Die Ergebnisse der ersten Studie zeigten keinen signifikanten Einfluss der Mitosehemmstoff-Behandlung auf den Regenerationserfolg der Mikrosporen-

Embryonen. Die beobachtete spontane und die durch Mitosehemmstoffe induzierte Diploidisierung sowie die direkte Konversionsrate der Mikrosporen-Embryonen war Genotyp-abhängig. Um eine erwünschte Diploidisierungsrate über 90% zu erreichen sind jedoch weitere Untersuchungen notwendig.

Ziel der zweiten Studie war die Optimierung der direkten Mikrosporen-Embryonen Konversionsrate zu Regeneraten, die eine direkte Keimung zeigten. Die direkte Sprossbildung aus den Mikrosporen-Embryonen ist häufig nur unzureichend, da ein hoher Prozentsatz an Embryonen aufgrund von sekundärer Embryogenese *in vitro* mehrfach subkultiviert werden muss. Dies bedeutet, dass die Sprossansätze wiederholt von undifferenziertem Gewebe freigeschnitten werden müssen bis eine Überführung in Erde und eine Regeneration zu Pflanzen möglich ist.
Im ersten Experiment dieser Studie wurden der Einfluss von zehn unterschiedlichen Sprossregenerationsmedien mit und ohne Phytohormone (Gibberellinsäure, 6-Benzylaminopurin, 3-Indolylbuttersäure) und eine 14-tägige Kältebehandlung bei 4 °C (Lichtthermostat) auf die direkte Mikrosporen-Embryonen Konversationsrate getestet. Die 14-tägige Kältebehandlung erfolgte sowohl unter acht Stunden Licht als auch in Dunkelheit. Die Standardkultivierung der Mikrosporen-Embryonen erfolgte im Kulturraum bei 26 °C und 12 Stunden Licht. Die fünf getesteten Winterrapsgenotypen zeigten 13% bis 39% direkte Sprossregeneration. Die höchste Rate von 43% wurde bei Kultivierung der Embryonen auf Gamborg B5-Medium mit 0.1 mg/L Gibberellinsäure erreicht. Die Kultivierung auf B5-Medium mit 0.1 mg/L 3-Indolylbuttersäure und 0.2 mg/L 6-Benzylaminopurin erzielte hingegen die niedrigste direkte Konversionsrate der Mikrosporen-Embryonen von 11%. Die Mittelwerte der direkten Konversionsrate aller Genotypen und Medien wurden durch die 14-tägige Kältebehandlung (28%) gegenüber der Standardkultivierung (14%) signifikant erhöht.
In zweitem Experiment wurden der Einfluss der vier effizientesten Spross-regenerationsmedien und eine 14-tägige Kältebehandlung bei 1.5 °C und bei 4 °C (Lichtthermostat) auf die direkte Konversionsrate untersucht. Die Kältebehandlung bei 1.5 °C erfolgte unter Lichtabwesenheit als auch unter acht Stunden Licht. Die Kältebehandlung bei 4 °C erfolgte dagegen in Dauerlicht und Dauerdunkel. Es wurden 13 unterschiedliche Genotypen getestet. Die Anzahl direkt gekeimter Mikrosporen-Embryonen variierte

zwischen 29% und 76%. Im Vergleich zur Kultivierung unter Standardbedingungen konnte mit der Kältebehandlung eine signifikante Erhöhung der direkten Konversionsrate der Mikrosporen-Embryonen erzielt werden (von 21% auf bis zu 71%). Nach vorheriger Kultivierung der Mikrosporen-Embryonen auf den unterschiedlichen Medien variierte die Keimrate zwischen 50% (MS) und 60% (B5 mit 0.1 mg/L Gibberellinsäure). Bei zwei von 13 Genotypen zeigten sogar alle untersuchten Mikrosporen-Embryonen direkte Sprossansätze, so dass eine Rate von 100% nach einer Kältebehandlung (1.5°C, Dauerdunkel) und Kultivierung auf B5-Medium mit Gibberellinsäure erzielt wurde.

Ergebnisse der zweiten Studie zeigten, dass trotz einer starken Genotyp-Abhängigkeit, die direkte Konversionsrate mit Kältebehandlung signifikant erhöht werden konnte. Die Wahl zwischen den besten Sprossregenerationsmedien nahm eine untergeordnete Rolle zur Verbesserung der direkten Konversion der Mikrosporen-Embryonen zu Regeneraten mit direkten Sprossansätzen ein. Abschließend lässt sich sagen, dass eine effiziente direkte Konversionsrate der Mikrosporen-Embryonen von über 70% für die meisten Genotypen bei einer Behandlung bei 1.5 °C im Dauerdunkel erzielt werden konnte.

Danksagung

An erster Stelle möchte ich mich ganz herzlich bei Herrn Prof Dr. Heiko Becker und bei Herrn Dr. Christian Möllers für die Überlassung des interessanten und vielschichtigen Themas bedanken.

Besonderer Dank gilt hierbei meinem Betreuer Dr. Christian Möllers für seine Anregungen und Hilfestellungen bei gleichzeitig gewahrtem Freiraum bei der Gestaltung und Durchführung dieser Arbeit, sowie für seine Unterstützung in allen Phasen der Promotionszeit.

Darüber hinaus möchte ich mich für die gewährte finanzielle Unterstützung bedanken. Das IGF-Vorhaben 16095 N FSt.1 der Forschungsvereinigung Gemeinschaft zur Förderung der privaten deutschen Pflanzenzüchtung e.V. - GFP, Bonn wurde über die AiF im Rahmen des Programms zur Förderung der Industriellen Gemeinschaftsforschung (IGF) vom Bundesministerium für Wirtschaft und Technologie aufgrund eines Beschlusses des Deutschen Bundestages gefördert.

Sehr viel Freude haben mir die Besuche bei den Pflanzenzüchtungsunternehmen bereitet, herzlichen Dank für die Einblicke in die Produktion, für die Kommunikationsbereitschaft und die interessanten Diskussionen, die mich bereichert, motiviert und zu neuen Ideen gebracht haben. Besonderer Dank hierbei an Dr. Jens Weyen, Dr. Annika Johrde, Clemens Springmann, Dr. Hanna Meier zu Beerenentrup , Dr. Peter Duchscherer, Dr. Monika Zuba, Dr. Stefan Abel und Simone Sendke.

Danken möchte ich Frau Prof. Dr. Traud Winkelmann für die Übernahme des Zweitgutachtens und Herrn Prof. Dr. Petr Karlovsky, der als weiteres Mitglied im Prüfungskomitee fungierte.

Ein besonderer Dank gilt außerdem Rosi Clemens, die mir immer mit Rat und Tat unterstützend zur Seite stand und Carmen Mensch, die unermüdlich die Ploidiemessung am Zytometer übernommen hat. Herzlichen Dank auch an Anna-Lena Schütz und Jenny

Ostermeyer für ihre Mithilfe. Außerdem einen großen Dank an Hans-Otto Heise, der mir in technischen Fragen sehr weitergeholfen hat und dem ich die Funktionsfähigkeit der Klimakammern zu verdanken habe. Ebenfalls danken möchte ich Wolfgang Link für seinen Rat bei den statistischen Fragen.
Besonders hervorzuheben sei mein Büro, die netten und freundlichen Menschen, die Freud und Leid mit mir geteilt haben, insbesondere Dank an Christopher, Daniela, Jörg, Makis, Mareile und Kathi.

Ein großer Dank an alle Mitarbeiter der Abteilung Pflanzenzüchtung des Departments für Nutzpflanzenwissenschaften der Universität Göttingen für die stetige Ansprechbarkeit und die freundliche Aufnahme ins Institut.

Mein größter Dank gilt meiner Familie und meinen Freunden, die mir in vielerlei Hinsicht unterstützend zur Seite standen und mich auch in schwierigen Zeiten motiviert haben. Ein Dank an meine Eltern, dass sie mir meine Träume ermöglicht und mir Mut gemacht haben meine Ziele zu verfolgen. Meiner Liebe danke ich für seine grenzenlose Unterstützung in den letzten 3 Jahren.

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