CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

FACULTY OF TROPICAL AGRISCIENCES

Optimization and Application of *in vitro* Techniques in Selected Members of the Family *Brassicaceae*

DISSERTATION THESIS

Author: Mgr. Alois Hilgert-Delgado

Department of Crop Sciences and Agroforestry

Co-supervisor: Ing. Miroslav Klíma, Ph.D.

Supervisor: Doc. Ing. Eloy Fernández-Cusimamani, Ph.D.

In Prague, August, 2016

ACKNOWLEDGEMENT

I would like to extent my heart-felt thanks to my supervisor, doc. Dr. Ing. Eloy Fernández Cusimamani, and to my co-supervisor, Dr. Ing. Miroslav Klíma, for their guidance, advice and support throughout my doctorate studies. They have provided me with constant support, encouragement and goodwill, helping and teaching me.

Special thanks belong to Dr. Ing. Iva Viehmanová for her help during my studies and laboratory work. I would like to acknowledge her as a great researcher and teacher. Her guidance, advice and collaboration definetely improved the main publication of my work. She, as co-author, dealed with the FCM analyses, and taught me about it and implemented bulked samples in the resynthesis verification.Similarly, I am very grateful to my colleagues from the Faculty of Tropical AgriSciences for their warm comradeship. I would like to thank the Czech University of Life Sciences, for supporting my studies and giving me a new platform to approach science.

Also I would like to very thank my close colleagues, Petra Bartošová and Václava Stresková for helpful assistance in laboratory in the Crop Research Institute, Prague and express my sincere gratitude to this place for letting me learn, work and growth while being welcomed during all this time. I would like to thank MSc. Vlasta Jarolímová for her assistance with plant material processing, Dr. Klara Kosová and MSc. René Miller for critical reviewing of the dissertasion thesis and linguistic revision.

I would like to express my heart-whole gratitude to my wife Ing. Jitka Najmanová for her support during my studies. I am happy to specially thank all my family, my esteemed and loved parents Carlos Hilgert Valderrama and Camila Delgado Coloma for their care, advice, and love, always encouraging me to do good things and the best I can, for their support and belief in me, together with my siblings always cheering up and supporting me through the distance. Finally, I am obligated to my dear aunt Dr. Nancy Hilgert for discovering my love for nature and inspiring me be a great scientist and to aim high.

This work was supported by the Czech University of Life Sciences in Prague, internal grant Project No IGA FTA 20145022 and by the Ministry of Agriculture of the Czech Republic, Projects No 0002700604, RO0416, QI111A075 and QJ1510172.

DECLARATION

I, Alois Antonín Hilgert-Delgado, declare that this thesis is my own work unless otherwise referenced or acknowledged, submitted for Ph. D. degree at the Czech University of Life Sciences Prague, Faculty of Tropical AgriSciences.

Prague, August 10th, 2016 .....

Mgr. Alois Hilgert-Delgado

TABLE OF CONTENTS

List of abbreviations

IAA	Indole-3-acetic acid
BAP	6-Benzylaminopurine
DAP	Days after pollination
DH	Doubled haploid
FA	Fatty acid
FCM	Flow cytometry
GC	Gas Chromatografy
HPR	Hybrid production rate
HSR	Hybrid siliquae ratio
MD	Differentiation medium
MR	Regeneration medium
MS	Murashige and Skoog (medium)
NIRS	Near Infrared Spectroscopy
NOR	Nucleolar Organizer Regions
RS	Resynthesized

List of figures

List of tables

Abstract:

This thesis is focused on the application and optimization of biotechnological methods within the family Brassicaceae. Most of this thesis is focused specifically the genus *Brassica*. The main objective of this dissertation thesis was to optimize and apply selected biotechnological methods in the resynthesis of oilseed rape (*B. napus*) from its baseline species (*B. oleracea*, CC and *B. rapa*, AA) and subsequently create valuable genetic plant materials.

The optimized procedure implemented in my work have exhibited better results of hybrid production (resynthesis) than in similar published experiments and provides a simplified and less laborious method via simple ovule culture and early hybrid verification.

The next accomplished step was to work with a wider range of materials using a broader source of different and contrasting genotypes from *Brassica rapa* (spring and winter oilseed and vegetable turnips) and *B. oleracea* (green and purple curly kales and cabbages) for new combinations and wider genetic diversity.

It was concluded that the optimized ovule culture protocol with early verification, developed in the framework of this thesis, is satisfactorily sufficient enough to be applicable in breeding programmes, aimed at diversity expansion of winter oilseed rape gene pool, as the resynthesized embryos were derived in most combinations.

New resynthesized verified lines were colchicine treated in order to obtain diploid regenerants and the fertile plants were self-pollinized and crossed with elite oilseed rape lines for further research and breeding.

Keywords: *Brassica napus* Resynthesis, Interspecific Hybridization, Flow cytometric analysis, Ovule Culture

Abstrakt: (Czech language)

Tato práce je zaměřena na aplikaci a optimalizace biotechnologických metod v rámci čeledi Brassicaceae. Většina z této disertační práce je zaměřena specificky na rod *Brassica*. Hlavním cílem této práce bylo optimalizovat a aplikovat vybrané biotechnologické metody v resyntéze řepky olejky (*B. napus*) z jejích základních druhů (*B. oleracea*, CC a *B. rapa*, AA) a následně vytvořit hodnotné genetické rostlinné materiály.

Optimalizovaný proces, který jsem realizoval ve své práci, vykazoval lepší výsledky než u podobných publikovaných experimentů a poskytuje jednodušší a méně pracný způsob s využitím jednoduché kultury nezralých vajíček a včasným ověřením hybridnosti.

Dalším krokem byl výzkum s širší spektrum materiálů - odlišných a kontrastních genotypů z druhů *Brassica rapa* (jarní a ozimé olejniny a zeleniny) a *B. oleracea* (zelené a fialové kadeřávky a zelí) pro nové kombinace k dosažení širší genetické rozmanitosti.

Optimalizovaný protokol kultury nezralých vajíček s včasným ověřením hybridnosti, který byl vypracován v rámci této práce, se ukázal být vhodným pro použití ve šlechtitelských programech, jejichž cílem je rozšiřování diverzity genofondu řepky ozimé, protože resyntetizovaná embrya byla získána z většiny kombinací.

Nové resyntetizované ověřené linie byly ošetřeny kolchicinem s cílem získat diploidní regeneranty. Fertilní rostliny byly samoopyleny v poupěti event. kříženy s výkonnými liniemi řepky olejné pro další výzkum a šlechtění.

Klíčová slova: Kultura nezralých vajíček, Resyntéza řepky, Mezidruhová hybridizace, Analýza průtokové cytometrie

1 INTRODUCTION

This thesis is focused on the application and optimization of biotechnological methods within the family Cruciferae (Brassicaceae), which is generally known as one of the ten most economically important plant families (Rich 1991). Most of this thesis is focused specifically on the genus *Brassica*. The crops of this genus are highly polymorphic and display enormous diversity (Warwick *et al.* 2009, Gupta *et al.* 2013) with 3,709 species and 309 genera (Al-Shehbazet *et al.* 2006 in Gupta *et al.* 2013). Their members are used as a source of fodder, feed, mustard condiments, green manure vegetables, and root and oil crops. In this thesis, different assessments of *B. napus, B. rapa* and *B. oleracea* were used in the experiments. *Brassica napus* belongs to the most important species of this genus. Widely, it is known in the form of edible rapeseed which has the synonym canola, from the canola oil brand, but it has a wider use for feed meal, industrial oil, meanly biofuel, and it is also used as a vegetable. Canola is the second most important oilseed crop and third largest source of vegetable oil in the world. *Brassica napus* L, despite the wide variety of purposes it can be grown for, it has been intensively bred in a relatively short period of time and thus exhibits a very limited genetic variability. Therefore, the resynthesized materials of *Brassica napus* L. from its common ancestors (different varieties of *B. rapa* and *B. oleracea*) by optimized protocols are one of the main achievements of the thesis. The use of these new genetic combinations for rape breeding programmes, hence, is applicable for needed un-conventional oil breeding approaches and also for specific oil profiles, vegetable uses, fodders, etc., and opens the doors to new breeding.

Additionally, the medium-sized family, Brassicaceae, includes the most studied model plant thale cress (*Arabidopsis thaliana*). Therefore, due to the economic value and the enormous quantity of studies in this family, there is a very good base for a wider application in research; the experiments can utilize well-known scientific approaches and the results can serve as welcomed scientific output with potentially economic impact and good chances for further improvement of applied *in vitro* techniques.

Improvement of success rates with a specific material is possible to achieve with the optimization of the treatment or technique. However, it is common to have different results from a certain technique and/or treatment from one species, and even variety, to another, due to different specific requirements like, for example frequency and quality, in protoplast fusion (Klíma *et al.* 2009) or diploidization rates, in microspore

culture (Klíma *et al.* 2008). Thus, it is important to constantly optimize treatments for specific plant materials and to keep track with new knowledge and constant improvement of technological methods and possibilities for their best ultimate combined application.

Techniques of *in vitro* cultivation are widely used for laboratory experiments of basic and applied research. They can be focused on specific plant studies moulding their environment to observe plant reactions and thus allowing to closely follow morphological and physiological development. Furthermore, because many of the external factors can be controlled, all the other factors still in very complex interactions can be better studied throughout *in vitro* cultivation. Hence, some conditions that would hardly be studied in nature can be better induced and studied in *in vitro* conditions.

We can also focus on in plant breeding by the selection of spontaneous or induced changes in the genome through mutations, changes of ploidy, hybridizations, trangenesis, etc. Breeders are elicited to study and inovate plant materials with such technologies. Helped by them, they are enabled to shorten the cultivation (breeding) cycle, and overcoming various incompatibilities while aiming to improve plant qualities and storage, healing of diseases, cryopreservation, micropropagation and/or microtuberization for *in vivo* production of ornamentals, field crops, etc. In many cases, the plant material of these purposes without *in vitro* cultivation techniques, cannot otherwise multiply, or at a lower rate, without desired characteristics, in a more expensive way, after a long period of time (when they reach sexual maturity for reproduction), etc. It is therefore possible to maintain alive, grow and multiply plants which would never survive at *in vivo* conditions as it is the case of some interspecific pollinated ovules, un-ripe embryos, protoplast fusions, regenerants after the application of normally unbearable stresses and, thus, thanks to these technologies and methods, we have got relatively free hands to better approach, select and implement plants mechanisms and functions. Thanks to this knowledge, mankind can benefit from improvements in agriculture through effective techniques to reach faster and better selection of needed traits in this fast growing and changing world.

2 LITERATURE REVIEW

2.1 *Brassica napus* and its Relatives

2.1.1. Taxonomical Introduction

Brassica genus belongs to the Brassicaceae family, inside the dicotyledons classification. Its genus members are informally known as cruciferous vegetables, cabbages or mustard plants. All the cultivated *Brassica* species are highly polymorphic including oilseed crops, root crops, and vegetables such as Chinese cabbage, Broccoli, and Brussels sprouts. These *Brassica* vegetables are dietary staple food in various parts of the world (Gupta 2013).

Most are seasonal plants (annuals or biennials), but some are small shrubs. The genus is native in most parts of Western Europe, the Mediterranean and temperate regions of Asia. Many wild species grow as weeds, especially in North America, South America, and Australia (Gupta 2013).

Classification for Kingdom *Plantae* down to Genus *Brassica* L.

Kingdom	*Plantae*	– Plants
Subkingdom	*Tracheobionta*	– Vascular plants
Superdivision	*Spermatophyta*	– Seed plants
Division	*Magnoliophyta*	– Flowering plants
Class	*Magnoliopsida*	– Dicotyledons
Subclass	*Dilleniidae*	
Order	*Capparales*	
Family	*Brassicaceae*	– Mustard family
Genus	*Brassica* L.	– Mustard

The following Table 1 presents an extensive classification by Gómez-Campo (1999).

Table 1 A taxonomic synopsis of *Brassica* with indication of subgenera, sections, species and subspecies

BRASSICA L.
Subgen. *Brassica*
Sect. *Brassica*
B. oleracea L.
B. montana Pourret
B. incana Ten. subsp. *incana*
B. i. subsp. *cazzae* (Ginz. and Teyb.) Trinajstic
B. villosa Biv. subsp. *villosa*

B. v. subsp. *bivoniana* (Mazzola and Raimondo) R.and M.
B. v. subsp. *drepanensis* (Caruel) Raimondo and Mazzola
B. v. subsp. *tinei* (Lojac.) Raimondo and Mazzola
B. rupestris Rafin subsp. *rupestris*
B. r. subsp. *brevisiliqua* Raimondo and Mazzola
B. r. subsp. *hispida* Raimondo and Mazzola
B. macrocarpa Guss.
B. insularis Moris
B. cretica Lam. subsp. *cretica*
B. c. subsp. *aegaea* (Heldr. and Hal.) Snogerup *et al.*
B. c. subsp. *laconica* Gustafsson and Snogerup
B. botteri Vis. subsp. *botteri*
B. b. subsp. *mollis* (Vis.) Trinajstic
B. hilarionis Post.
B. carinata Braun
B. balearica Pers.

Sect. *Rapa* (Miller) Salmeen

B. rapa L. subsp. *rapa*
B. r. subsp. *campestris* (L.) Clapman *B. r.* subsp. *chinensis* (L.) Hanelt
B. r. subsp. *dichotoma* (Roxb.) Hanelt *B. r.* subsp. *narinosa* (Bailey) Hanelt
B. r. subsp. *nipposinica* (Bailey) Hanelt *B. r.* subsp. *pekinensis* (Lour.) Hanelt *B. r.* subsp. *trilocularis* (Roxb.) Hanelt
B. napus L.
B. juncea (L.) Czern.

Sect. *Micropodium* DC.

B. fruticulosa Cyr. subsp. *fruticulosa*
B. f subsp. *djafarensis* Blanco and Matarranz
B. f subsp. *dolichocarpa* Emberger and Maire
B. f subsp. *glaberrima* (Pomel) Maire
B. f subsp. *mauritanica* (Cosson) Maire
B. f. subsp. *numidica* Maire
B. f subsp. *pomeliana* Maire
B. f subsp. *radicata* (Desf.) Maire
B. nigra (L.) Koch
B. cossoniana Boiss. and Reuter
B. spinescens Pomel
B. maurorum Durieu
B. procumbens (Poiret) O.E.Schulz
B. cadmea O.E.Schulz
B. desertii Danin and Hedge

Sect. *Brassicoides* Boiss.

B. deflexa Boiss.

Sect. *Sinapistrum* Willkomm

B. barrelieri (L.) Janka
B. oxyrrhina Coss.
B. tournefortii Gouan

Subgen. *Brassicaria* (Godr.) Gómez-Campo

Sect. *Brassicaria* (Godr.) Cosson

B. repanda (Willd.) DC. subsp. *repanda*
B. r. subsp. *africana* (Maire) Greuter and Burdet
B. r. subsp. *almeriensis* Gómez-Campo
B. r. subsp. *blancoana* (Boiss.) Heywood
B. r. subsp. *cadevallii* (Font Quer) Heywood

B. r. subsp. *cantabrica* (Font Quer) Heywood
B. r. subsp. *confusa* (Emb. and Maire) Heywood
B. r. subsp. *galissieri* (Giraud) Heywood
B. r. subsp. *glabrescens* (Poldini) Gómez-Campo
B. r. subsp. *gypsicola* Gómez-Campo
B. r. subsp. *latisiliqua* (Boiss. and Reut.) Heywood
B. r. subsp. *nudicaulis* (Lag.) Heywood
B. r. subsp. *saxatilis* (DC.) Heywood
B. r. subsp. *silenifolia* (Emberger) Greuter and Burdet
B. desnotesii Emb. and Maire
B. gravinae Ten.
B. jordanoffii O.E.Schulz
B. loncholoma Pomel
B. nivalis Boiss. and Heldr.
B. elongata Ehrh. subsp. *elongata*
B. e. subsp. *integrifolia* (Boiss.) Breistr.
B. e. subsp. *imdrahsiana* Quezel
B. e. subsp. *pinnatifida* (Smal'g) Greuter and Burdet
B. e. subsp. *subscaposa* Maire and Weiller
B. setulosa (Boiss. and Reuter) Cosson
B. somaliensis Hedge and Miller

Sect. *Nasturtiops* (Pomel) Salmeen

B. souliei (Batt.) Batt. subsp. *souliei*
B. s. subsp. *amplexicaulis* (Desf) Greuter and Burdet
B. dimorpha Coss. and Dur. (Gómez-Campo, 1999)

For the taxonomic synopsis of its allied genera see Apendix B

The classification within and outside the genus may vary among time and botanists statuses. Some related species to the genus were formerly part of it: *Hirschfeldia incana* also known as *B. geniculata* (hoary mustard), white mustard *Sinapis alba* - as *B. alba* or *B. hirta and Sinapis arvensis* – as *B. kaber* (wild mustard or charlock). The cited author mentions that some subspecies categories are applied more traditionally than logically, as it is the examble of some *B. napus* cultivars such as *napobrassica, rapifera, pabularia*, etc (not included in the table). A complete list of species, subspecies and varieties individually can be seen in following link:

https://grinczech.vurv.cz/gringlobal/taxonomybrowse.aspx

For comparison of different classifications, only 19 Species and 12 accepted taxa in *Brassica* are accepted according to the United States Department of Agriculture:

http://plants.usda.gov/java/ClassificationServlet?source=display&classid=BRASS2

2.1.2 Importance, Properties and Uses

Brassica crops are well known in the agricultural and horticultural sector and thus there is also high scientific interest in this genus. Breeding of different cultivars has

developed into food from roots (rutabaga, turnips), stems (kohlrabi), leaves (cabbage, collard greens), flowers (cauliflower, broccoli), buds (Brussels sprouts, cabbage), and seeds (many, including mustard seed, and oil-producing rapeseed). Some forms with white or purple foliage or flowerheads are also grown as ornamentals in Asia (personal observations).

Brassica oil crops are very important crop plants. *Brassica napus* is the most important species of Brassicas. Its seeds represent the world's third most important source of vegetable oil with 71 million metric tons and their use is continuing to grow. Only palm oil, although not produced from a seed, and soya bean provide more oil in the world. Rapeseed oil production is followed by sunflower-seed, flax-seed, cotton-seed and peanut (USDA; USDA January 2015).

Rapeseed contains both high oil and protein content. It is the second largest feed meal after soybean meal, and, additionally to the oil for human consumption, in the European Union and Eastern Europe, it is the natural feedstock for biodiesel because of its availability as the primary oil crop and local legislation. A small amount of rape is produced for HEAR oil (high-erucic acid rapeseed oil) used in a variety of industrial and consumer products (USDA), and as a vegetal (rutabaga). In the Czech Republic, it is the most widespread oil plant and the second most frequently cultivated crop after cereals.

Global rapeseed production has witnessed a steady upward movement during the past 40 years, rising from the sixth largest oilseed crop to the second largest one, which makes up 15% of global consumption of vegetable oil (Statista 2015). The seed contains approximately 38 to 45 percent oil and the remainder consists of rape meal and a small amount of waste. Thus, it is the second largest protein meal produced in the world, although its productions is five times lower than soybean meal, which has a higher protein content (USDA).

2.1.3 Botanical Description of Selected *Brassica* Crops

2.1.3.1 Oil Rapeseeds

Rapeseed or rape name is generally used for both species; *Brassica rapa* variety *oleifera* and *Brassica napus* variety *oleifera*. Another name for *Brassica rapa* is *Brassica campestris*, and it is commonly found in older literature. These rapes have a lengthy tap root with many horizontal lateral branching. Rapes have well branched stems as well. However this branching is dependent on population diversity or proximity. This also

affects the height in which it starts branching. The stems are usually round and upright and usually green but turning yellowish with an increase in age. Stem heights vary but are 80-150 cm in most areas. However, there are areas were rapeseed grows as high as 250 cm. On the other hand, this made the crops difficult to harvest; therefore dwarf varieties are popular in such areas (Weiss 1983).

The leaves of the rapeseed plant are dark green, pinnate on the lower and lanceolate, sessile and clasping the stem. The two species are distinguished phenotypically in the field once the stem elongates by the small difference in how close the leaves clasp the stem. As for *B. rapa*, the upper leaves clasp the stems closely and are auriculate. *B. napus* is distinguished by only the lower leaves clasping. *B. napus* is more glabrous and glaucous, its beak is thicker and its leaves are only loosely embracing (Gómez-Campo, 1999). On the main stem the number of leaves can be between five and twelve on spring type and to forty or more on winter type. The inflorescence is originated from the terminal end of the main stems and branches. These carry very bright yellow flowers. Flower numbers can be influenced by many factors such as variety climate and farming techniques. The number of flowers on one plant may be from 12-25. Although this sound like a lot it produces more flowers than pods and generally 65-70 percent of these flowers become pods and the rest are discarded. Low temperatures for vernalisation are generally needed for flowering in winter type. This period may last from 3-5 weeks or longer (Kershaw 1998).

A common loss of yield is caused by loss or destruction of buds. Hail and insects are a major cause bud loss. The major insects that cause this bud loss are pollen beetles (i.e. *Meligethes aeneus*) and seed weevils (i.e. cabbage seedpod weevil, *Ceutorhynchus assimilis* providing oportunity secondarily to *brassica* pod midge, *Dasineura brassicae*). The rapeseed fruit consists of a pod that is long and narrow, approximately 5-10 cm in length. These consist of two carpels divided by a false septum which when mature shatters. The characteristics of the pods vary widely from thick walls, number of carpels and shattering or non-shattering. Surprisingly enough is the fact that several different pod types may be found on one plant. These pods may contain fifteen to forty small round seeds that are about 1-2.5 mm long. Often the seed coat is rough and pitted. These seeds are dark brown to black in color. The protein content of these seeds range from 10-45% and the oil content ranged from 30-50%. This oil that is liberated is dark in the crude form but when refined it turns to light yellow color resembling sunflower oil. This species contains little

endosperm. The embryo is made up of two conduplicated cotyledons. These are oil and protein rich strains which are much like the aleurine cells that lie just under the seed coat. The rapes' center part of the seed consists of meristematic tissue. This is where the epicotyl, hypocotyl and the radicle originate.

It is known that rapeseed crop does better in well drained fields with aerated soils. A modest amount of rainfall of about 450-500 mm during the flowering stage is ideal. Anything over 700 mm would be harmful to the crop because of water logging of fungal infection. It has been found that rape is tolerant when it comes to salinity of the water (Appelquist & Ohlson 1972).

Rapes are grown in heavy clay, volcanic ash and even fight sandy type soils. Almost any soil type may be used except those that crust over or as mentioned earlier those that are soggy or water logged. The pH of the soil is variable as well and can range from 5-8. Basically, most experts say that soil that is good enough for wheat is good enough for rape. This crop is also able to withstand not only many types of soils but a great range of photoperiod length as well. There has been rape grown above the Arctic Circle during periods of 24 hours of daylight and also grown in areas with 8-10 hours of light (Kershaw 1998).

Initially, rapeseed was grown in the northern and southern hemispheres where the climate is temperate. However, with all of the selective breeding of rape, it has become more and more adapted to wider ranges of climates. Its range as a cultivated crop includes Canada, United States, Europe, Asia, the former Soviet Union, China, Japan, India, South America, Australia, South Africa and many other smaller countries. It is even grown in some higher elevations in the tropics.

Fertilizers are used to increase the yields of rape. Organic manure and chemical fertilizers all are useful in increasing yield. It has been found that the phosphorous and potassium need in rape is generally the same as for that of wheat or other field crops. However, rapes need for nitrogen is much greater than for most crops. The most important thing about fertilizer use is the time of application. Other important nutrients are calcium, sulfur and boron (Kershaw 1998).

The plant is ready for harvest when the pods and stern become yellow and the seeds very dark. These seeds rattle in the pod when shaken. The moisture of these seeds should be about 15%. The date of harvest is variable but in general it is 85-125 days after sown for spring rape and 180-240 days for winter rape. There is an about one week time period

when the rape ripens and must be harvested for the greatest yield. Many times this crop is wind rowed to avoid shatter loss. This is not as easy as rape tends to bunch up after it is cut which makes it difficult to combine. Windrowing is best done when the seed pods are yellow halfway up the stalk and the seeds have a chocolate color. Because the high oil content and small seed size, rape must be handled quickly and carefully during its drying and storage. Rapeseed must be dried before storage. After this they may be used for oil production or meal (Kershaw 1998).

Rape oils have high nutritional value and cost competitiveness in comparison with other leading vegetable oils. The American Heart Association has recommended a reduction in saturated fat intake into the diet because of direct links between this and chronic heart disease. Canola oil is the lowest in saturated fat of any oil on the market. It only contains 6% saturated fat and is high in monounsaturated fat. This is 50% less saturated fat than corn oil (Weiss 1983). In fact the most popular low saturated fat oil is called Puritan oil, which is 100% canola oil. Canola's oil quality is high as it is stable in heat and light in addition to have a bland flavour, making it perfect for products which cannot tolerate flavor that is carried through from the cooking oil.

2.1.3.2 Vegetables

Turnips or white turnips (*B. rapa* subsp. *rapa*) are root vegetable in the Cruciferae family know for its bulbous taproot and it is mainly used as an ingredient in soups and stews. This biennal plant which often is a mixture of the colors purple, white, and/or yellow (Polidoro, 2008). There are many varieties of turnips, and each has a different flavor and storage capacity. These biennal plants grow in similar patern to carrots, growing the first year with leafes growing above the ground and they can be harvest after one year of growing before flowering and set seeds. Small varieties are planted for human consumption and larger varieties for livestock. Technically this root vegetable is not actually a root, but a "swollen stem which grows beneath the surface of the soil" and it is commonly grown in temperate climates worldwide. The rutabaga is a different species which while similar to turnip, it is actually a cross between the turnip and cabbage, hence a *Brassica napus* vegetable.

While they probably are indigenous from the West Mediteranean region to central Asia their true antiquy is not well documented (Gómez-Campo 1999). Romans ate turnips;

however, they became widespread only in 18th century. More details about turnips can be found in Polidoro (2008).

Brassica oleracea L. grows wild in the Atlantic coasts of Europe, where it might have been cultivatd by Celts in tis primitive form (kales). When it was brought to the East Mediterranean region it became fully domesticated and started an explosive diversification. Among some widely known forms we can distinguish kales which develop a strong main stem and are used for their edible foliage (curly kales, stem kales Jersey kale), Cabagges which are characteriye by formation of heads of tighty packed leaves (head Cabbage, saviz cabbage abd brusseks sprouts), kolhrabi grown for its thickeded stem, inflorenscence kales, with their edible inflorescences (cauliflower, broccoli calabrese) and Chinese alboglabra kale, used for its leaves. To summarize, *Brassica oleracea* varieties have a very diverse botanical description since each modern *Brassica* vegetable is considered typical for its special characteristics, mostly developed by rather different goals and priorities of individual breeding programs (Gómez-Campo, 1999). The vast array of crop types in *Brassica Oleracea* has led to acceptance at the subspecies and variety levels of descriptions based around the specialized morphology of the edible parts and habits of growth within the crop types (Wellington & Quartley, 1972: in Dixon 2007). Therefore this chapter only discribe generally the used crop types in the thesis, for resynthesis, Curly Kales, *B. Oleraceae* convar. *Sabellica*, known for their winter hardiness, and also accessions of Cabbages and Sprouts, which are also biennal plants.

Kales are ancient cole crops, closely related to the wild forms of *B. oleracea*, and many distinctive types were developed in Europe, closely resembling their wild cabbage progenitors. In borecole or curly kale, the leaves are crinkled and more or less finely divided; often green or brownish-purple, and they are used as vegetables. The stem is coarse, neither branched nor markedly thickened, and 300–1000 mm tall (some varieties taller when flowering). Curly Kales are not worldwide known, but my observation is that they are becoming economically more important.

Heading cabbages are the popular image of vegetable brassicas in Europe. From the 16th century onward, European colonists introduced cabbages worldwide. Cabbages are biennial herbs 400–600 mm tall at the mature vegetative stage and 1.5–2.0 m tall when flowering in the second year. Mature plants have a ramified system of thin roots, 90% in the upper 200–300 mm of the soil, but some laterals penetrate down to 1.5–2 m deep. Stems are un-branched, 200–300 mm long, gradually thickening upward. The basal leaves

form in a rosette of 7–15 sessile outer leaves each 250–350 × 200–300 mm in size. The upper leaves form in a compact flattened globose to ellipsoidal head, 100–300 mm in diameter, composed of a large number of overlapping fleshy leaves around the single growing point. These leaves are grey to blue-green, glabrous, coated with a layer of wax on the outside of the rosette, and light green to creamy white inside the head, especially with white-headed cabbage. The inflorescence is a 500–1000 mm bractless long raceme on the main stem and on axillary branches of bolted plants (Dixon 2007).

2.1.4 Origin and Evolution

Early records indicate that Brassicas had been cultivated for several thousand years in Asia. *B. rapa* had a wide distribution before the recorded history (Gupta *et al.* 2013). Seed of both *B. rapa* and *B. juncea* were found in the archaeological excavation of ancient village Banpo, China, that existed in Neolithic times 6,000–7,000 years ago (Liu 1985). Domestication of rapeseed in Europe appears to have started in the early Middle Ages. *B. rapa* initially spread mainly as turnip rape crop within Europe. Rape seed was predominant during the thirteenth century. The rapeseed oil was used as a major source of lamp oil and it was replaced by petroleum by the end of nineteenth century. In the 1970s canola emerged with high quality oil and meal for both human and livestock consumption. Since then, it is one of the most important oilseeds in moderate climate worldwide.

Brassica oil crops grow at relatively low temperature. Species grown as oilseed crops are *Brassica napus* and *B. rapa* (Rapeseeds) which are grown as both winter and spring types mainly in temperate regions while spring types join *B. juncea* (Indian mustard) and *B. carinata* (Abyssinian or Ethiopian mustard) in subtropical parts of Asia as the main source of oil (Gupta *et al.* 2013). Meanwhile semi-winter type of *B. napus* occurs in China, which is not compatible to the conditions suitable for European winter types. The spring type is generally grown in northern countries in order to avoid the long winters; however, winter types sown in autumn produce higher oil yield in temperate climates, for example in the Czech republic .

Besides oil crops, the vegetable *Brassica* include *B. napus, B. rapa* (Chinese Cabbage, Pak-Choi, Chinese mustard, Broccoli and Kale); *B. oleracea* (Cabbage, Broccoli, Cauliflower, Brussels sprouts, Kale, etc). *Raphanus sativus* and *Lepidum sativum*, *B. nigra* (Black mustard), *B. juncea*, (Brown mustard) and *Sinapis alba* are the main condiment of crops.

2.1.5 Genetic Diversity

As Crucifers have played a great role in the human history by contributing a good share of food in one form or another, including medicinal type, their domestication and thus their modification, diversification and improvement occurred along with human classification of many wild and cultured species around the globe, many times leading to inconsistency in names due to the many forms in one species and their evolution in different places of the world (Gupta *et al.* 2013). On the other hand, the effect of long and intensive breeding on on specific agronomic traits leads to a bottle neck effect which force breeders to go back to their ancestors for lost, mutated or acquired genes in the pursuit of gene sources of resistance to current biotic and abiotic stresses, higher yield and other agronomically desired characteristics. This phenomenon is emphasized in species whose origin had limited geographic range and a restricted gene pool from which new (broader) genetic variability is limited (Girke *et al.* 2012, Rahman 2013). This is the example of the three amphidiploids (syn. allodiploids) synthetized *B. carinata* (*B. nigra* + *B. oleracea*), *B. juncea* (*B. nigra* + *B. rapa*) and *B. napus* (*B. rapa* + *B. oleracea*) in the genus *Brassica*. These three species evolved by the combining of chromosomes of the other three parental species forming a part of the triangle of U (Fig. 1). Currently, the improved oilseed *Brassica napus* with zero erucic acid and low glucosinolate levels bred from genetic variations within the population of *B. napus* and *B. rapa* is called ‘‘Double Low (00) rapeseed’’ or ‘‘canola’’. The name canola is a contraction of Canada and ola, meaning oil (http://www.canolacouncil.org/oil-and-meal/what-is-canola/); however, other sources as The Free Dictionary claim it stands for "Can(ada)+o(il)+l(ow)+a(cid). Initially, many other important agronomic traits of the first “00” or Canola cultivars were poorer than the traditional types of rapeseeds. While intensive breeding efforts in the past few decades increased seed yield as well as other agronomic traits (Rahman 2013), however, it also resulted in narrowed genetic diversity of breeding materials (Girke *et al.* 2012, Rahman 2013). The basic aim of oilseed rape breeding focuses not only on the traditional production of high-yielding varieties with satisfactory agronomic characteristics (resistance to diseases, frost tolerance, earliness, shatter-resistance, etc.) and the most important quality parameters of rapeseed – high oil content, but also on the specific composition of fatty acids profile in the oil and increased resistance to major diseases and other biotic and abiotic stressors. Due to continuing climate changes that accompany the increasing

occurrence of droughts, torrential rains and atypical winter periods, it becomes also important to achieve the adaptability of varieties to specific soil and climatic conditions.

Successful breeding of competitive varieties of both spring and winter oilseed rape requires constant innovation of breeding programs and the introduction of modern biotechnological processes. Nowadays, the loss of genetic diversity is a concern to both private and public canola breeders and researchers as diversity is critical not only for improvement of the crop for seed yield and agronomic properties, but also for protecting the crop from new diseases and pests. Increased number of plantations and the introduction of new types of varieties are related to an emphasis on disease and pest resistance including those for less widespread varieties. It is also crucial to approach the genetic potential of relatives of the crop species of *Brassica* and allied genera (members of the family *Brassicaceae*) for the establishment of long-term breeding programs of these crops. They represent a source of agronomic traits including cytoplasmic and nuclear male sterility, resistance to disease and insect and nematode pests and tolerance to cold, salt and drought conditions (Warwick *et al.* 2009). The danger of genetic uniformity among the cultivars in an agro-ecological region is well documented, for example, disasters from late leaf blight disease in potato in Ireland, and southern blight disease in corn cultivars in the United States (Rahman 2013). Therefore, scientists have to employ biotechnology to introgress favourable germplasm of other species and create improvement as well as diversity, which is limited by means of traditional methods, but can be obtained by using advanced *in vitro* techniques. In addition, these methods can contribute to significant shortening of the breeding process.

For operative extension of the rapeseed genetic diversity we can currently apply a range of biotechnological methods: mutagenesis, somatic hybridization, genetic manipulation or distant hybridization between *B. rapa* and *B. oleracea* subspecies and varieties, combined with *in vitro* procedures. Mutagenesis and somatic hybridization are random processes, in which the genetic, respectively, genomic composition of the resulting hybrid can not be predicted (Wang *et al.* 2008); Genetic manipulation is limited by the availability of positive genes that control desired traits, considerable financial demands, transformation techniques, subsequent analysis and especially limited cultivation of genetically modified plants in the EU countries. The latter method is suitable for cultivation of immature eggs (ovules), regeneration of embryos under aseptic conditions (ie. embryo rescue) and chromosome doubling in order to directly create - resynthesize –

in genetic terms, the allotetraploid *Brassica napus* L. Although rape resynthesis is already established method, currently, significant accomplishments were achieved by optimizing biotechnological processes during the course (Hilgert *et al.* 2015; Karim *et al.* 2014; Sosnowska & Cegielska-Taras 2014). Improving the yields of hybrid production while simplifying the entire process of the method are aimed to make more accessible its routine use including in for the breeding of domestic varieties of rapeseed.

2.2. Hybridization of Selected *Brassica* and Allied Genera

Many researches have been directed to transfer genes between *Sinapis alba* and *Brassica carinata* and to study the species relationship. The genus *Sinapis* together with *B. nigra* is cultivated as a source of mustard condiments and exhibits several useful agronomic traits such as resistance to herbicides, insect and nematode pests and tolerance to drought conditions (Warwick 1993). Three botanical species, namely *B. nigra*, *B. juncea* and *B. carinata* in the genus *Brassica* together with *S. alba* are known as mustard crops. *Brassica* contributes a pungent and *S. alb*a contributes a hot principle (Hemmingway 1976). *Brassica* contains genetic information to produce allylglucosinolate and *Sinapis* contains information to produce p-hydroxybenzyl glucosinolate in its genome. Intergeneric hybrids among some species of *Brassica* and *Sinapis* have been attempted intensively in the early nineties (Mohapatra & Bajaj 1987; Ripley & Arnison 1990; Banga & Labana 1991; Inomata 1991, 1992, 1994; Sridevi & Sarla 1996). The latter was the last hybridisation attempt between *B. carinata* and *S. alba* and was unsuccessful. After that *B. nigra* and *S alba* were successfully crossed (Choudhary & Joshi 2000) and other four hybrids from *Brassica* and *Sinapis* species were obtained by Momotaz *et al.* (1998) among few other attempts as reported by FitzJohn *et al.* (2007). Intergeneric hybridisation followed by chromosome-doubling of sterile hybrids leads to production of allopolyploids. Alloploidy has been successfully exploited in plant breeding by synthesizing new species that could be used as new crops. Usually, it is difficult to produce such hybrids due to cross-incompatibility barriers. Development in biotechnology and embryo rescue techniques may provide new genetic variability for the breeding of cruciferous crops (Mohapatra & Bajaj 1987; Seyis *et al.* 2003; Girke *et al.* 2012; Zhang *et al.* 2011; Rahman 2013). In general, a series of studies have been undertaken to exploit the following objectives, such as to assess the interaction of the two species' genomes (*B* and *S*) in the expression of allylglucosinolate and p-hydroxybenzyl glucosinolate together in one

combined genome, and to produce new amphidiploids with p-hydroxybenzyl glucosinolate of AASS and CCSS combinations.

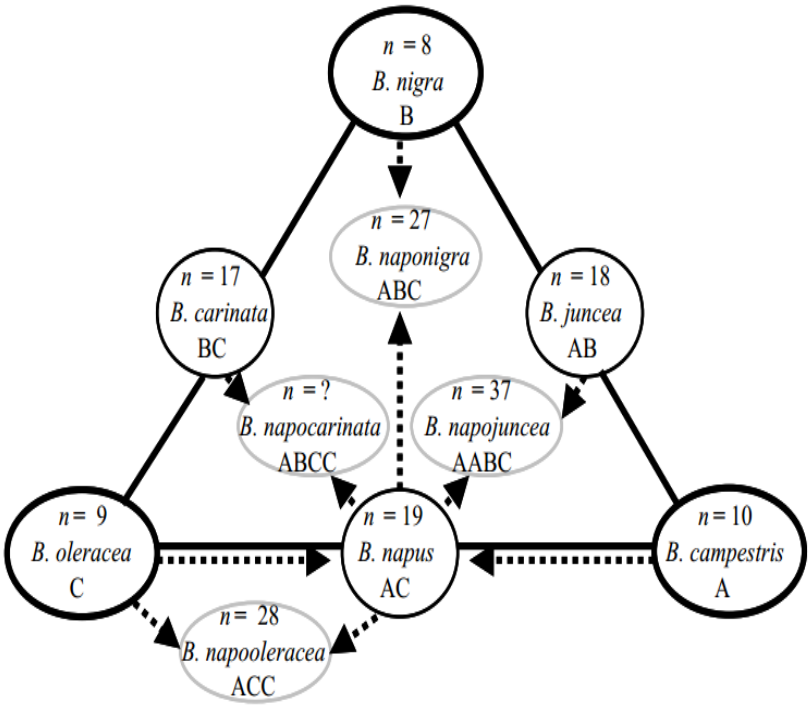


Fig. 1 Hybrid combinations between unrelated species in *Brassica*. The continuous lines represent amphidiploid products of sexual recombination of diploid progenitors *B. oleracea*, *B. rapa* and *B. nigra*. Dashed arrows represent the successful combinations of somatic hybrids leading to the formation of composite genomes (Figure from Navrátilová *et al.* (2004)).

2.2.1 *Brassica napus* L. and its Ancestors

Breeders are continuously engaged in improving this oilseed and biofuel crop species yield and quality parameters. Oilseed rape appeared approximately ~7500 years ago (Chalhoub *et al.* 2014) through the natural hybridization of two *Brassica* species. Despite its importance nowadays, it has been domesticated during the past 400-500 years only (Prakash *et al.* 2011). Dual bottle necks of polyploidy and intensive domestication implied to this crop a narrow genetic base (Chatterjee *et al.* 2016). Unlike several other important crops, sources of genetic variation from natural populations cannot be used (Prakash & Hinata 1980). This fact has elicited scientists to use non-traditional approaches to introgress traits from allied species (Rahman *et al.* 2011).

2.2.2 The Triangle of U and *Brassica* Resynthesis

As the relationship between AA and CC genomes and the possibility to create amphidiploids (i.e. allodiploids) *B. napus* (AACC, n = 19) de novo from interspecific crosses between diploid *B. rapa* L. (AA, n = 10) and *B. oleracea* L. (CC n = 9) was revealed (U 1935; see fig. 1), far-reaching opportunities to expand variability in this way were opened. The resynthesis of *B. napus* assisted by biotechnology can quickly provide essential basic germplasm for further improvements, broadening genetic variability and introduction of important agronomic traits (Seyis *et al.* 2003; Zhang *et al.* 2011). Thus, resynthesis of *B. napus* has become an intensively studied topic nowadays (Rahman *et al.* 2011), and, therefore, it is not surprising that numerous attempts to create *B. napus* with desired properties from its diploid progenitors have been reported (Seyis 2013).

Recently, to broaden the diversity within *Brassica napus* gene pool, studies on resynthesis of rapeseed through crossing the selected subspecies of *Brassica rapa* L. and *Brassica oleracea* L. and obtaining plants through *in vitro* culture of isolated embryos in the early stage of their development have been published (Rahman 2005, Wen *et al.* 2008, Sosnowska *et al.* 2010). It has been proven that resynthesized plants are distinct from cultivars of winter oilseed rape which are bred and cultivated nowadays (Sosnowska *et al.* 2010), so they would serve as sources of new genetic variability in rapeseed breeding programmes.

Indeed, not only the resynthesis of *B. napus* alone, but all crop species from the genus *Brassica* became a model system to track early genome changes following allopolyploidization (Xu *et al.* 2012). Xu *et al.* (2012) also provide the confirmation of genetic changes following hybridisation and genome doubling in synthetic *B. napus* (two necessary processes for amphidiploids formation) emphasized that different pathways may contribute to new genetic diversity. Furthermore, hybridisation could induce “genomic shock” and a large-scale reprogramming of the genome structure as polyploidy formation pathways differ in their genetic outcome (Szadkovski *et al.* 2011).

Interestingly, not intergeneric, but complicated interspecific crosses within the genus *Brassica* have been more popular for exchange of genomic information in *B. napus* lines. This has been accomplished through substitution of its A genome with the A genome of *B. rapa* and *C* genome with the *C* genome of *B. carinata* through the development of several trigenomic hexaploids (*AABBCC*) from crosses between different *B. rapa* and *B.*

carinata lines, and crossing the hexaploids to natural *B. napus* and selection for *B. napus* plants with the *A* genome content of *B. rapa* and *C* genome content of *B. carinata*. (Li *et al.*2004, 2006; Xiao *et al.* 2010). According to the results in Xiao *et al.* (2010), the *B* genome chromosomes are eliminated relatively easily during self-pollination of this interspecific hybrid; thus, *B. napus* lines containing more than 75% of the genomes of *B. rapa* and *B. carinata* can be achieved. These reconstituted *B. napus* lines in test hybrids with different natural *B. napus* showed high-parent heterosis in the majority of cases, and seed yield in the hybrids was positively correlated with the proportion of the *A* and *C* genomes of *B. rapa* and *B. carinata* in these reconstituted lines (Li *et al.* 2006).

For a complete database approach assembled on reproductive compatibility for eight crop species in *Brassica, Raphanus and Sinapis*, using data from hand pollination, spontaneous (unassisted) pollination and trials using *in vitro* techniques of more than three hundred listed studies and two hundred species combinations, see FitzJohn *et al.* (2007).

Currently, there are two basic techniques that can be used to obtain resynthesized genotypes:

2.2.3 Resynthesis from Protoplast Cultures

Chen and Heneen (1989) reviewed optimistically results of some works where resynthesized hybrids were obtained by protoplast fusions as a powerful and futuristic alternative technique in *Brassica* spp. breeding. Although the somatic hybridisation through the protoplast fusion technique has been used to construct resynthesized (RS) rapeseed (i.e. Ozminkowski & Jourdan 1994), it is a random genomic recombination process and the resulting genome composition of the somatic hybrids is not well known (Wang *et al.* 2008). Now, this technique is more widely applied for intergeneric crosses with *B. napus* in order to increase germplasm variability (Heath & Earle 1996; Wang *et al.* 2013) or to introgress specific traits controlled by genes in the cytoplasm or their combination (Navrátilová *et al.* 2004). It is worth mentioning that authors agreed that although this technique helps to circumvent cases of unviable crosses, the realization is exhaustive and of low efficiency. Hence it is not suitable to use where embryo or ovule culture techniques can substitute it unless there were aims of recombining specific cytoplasm such as some cytoplasmically-controlled agronomic traits (Wojciechowski 1985b). It also has great variability as somatic hybridisation can generate more genetic

variation than sexual hybridisation or provide with new cytoplasmic recombination which can be desired (Heath & Earle 1996, Yadav *et al.* 2009).

2.2.4. Resynthesis from Intespecific Crosses

2.2.4.1 Interspecific Crosses of *B. rapa* with *B. oleracea*

A widely used method for resynthesis of oilseed rape involves crossing of *B. rapa* with *B. oleracea* in flower buds emasculated before anthesis in order to avoid self-pollination and possible pre-fertilization barriers, rescuing the resulted embryos at early stages of development and regenerating whole plants on an artificial nutrient medium. This is usually achieved by *in vitro* cultivation of ovaries, young ovules or excised embryos to overcome post-fertilization barriers (i.e. endosperm failure, inhibited nutrient input, embryo degeneration, hybrid inviability and weakness) (Van Tuyl & De Jeu 1997; Bhat & Sarla 2004). Resynthesis of *Brassica napus* and interspecific crossing in general are laborious and time demanding (Akbar 1989). Interspecific crosses produce low embryo yield and can vary according to genome similarity regarding genetically distant species or genotypes (Olsson 1960). Resynthesis causes new amphidiploid nuclear-cytoplasmic interaction, which contributes to new alterations and broadens the evolutionary scene of oilseed rape (Song *et al.* 1993) and, at least in part, have a plasticity in genome response which may be advantageous for adaptation and rapid establishment in different niches (Xu *et al.* 2012). On the other hand, these interactions may lower yield because of possible nuclear-cytoplasmic incompatibility (Cui *et al.* 2012) or genetic incompatibility which may just completely block sexual hybridisation (Wang *et al.* 2013).

Bhat and Sarla (2004) examined the causes of variation (i.e. in pollination efficiency and pollen tube entry) during interspecific hybridisation, and found important effects on both parental genotypes. The substantial effect of a genotype on embryo yield has been observed in a number of articles (Lu *et al.* 2001; Bhat & Sarla 2004; Malek *et al.* 2012; Wen *et al.* 2008). Several papers have also reported notorious parental influence on seed and siliquae setting in interspecific crosses (Momotaz *et al.* 1998; Wang & Campbell 1998; Lu *et al.* 2001; Malek *et al.* 2012). Furthermore, a substantial part of the variability is caused by a combination of physiological differences among plants during pollination cycles and bud age (gynoecium immaturity of younger buds) and the emasculation effect as reported by Brown *et al.* (1991). Implicated wounding and damage of tiny *B. rapa*

gynoecium/ovary may also occur during emasculation and bud pollination. For the latter reason, the use of *B. oleracea*, with (on average) bigger buds and ovaries, as a female component seems to be more feasible option (Brown *et al.* 1991). Another source of variability might be connected with the siliquae containing non-pollinated ovules or the individual differences between siliquae. For example, developed siliquae with only one pollinated ovule was considered "fully pollinated" as it is difficult to accurately distinguish between undeveloped, already pollinated, and non-pollinated ovules during routine isolation after short periods following pollination. Therefore, the numbers of pollinated ovules probably vary between siliquae. Besides the above mentioned reasons, overall differences can also be hampered by different requirements of individual genotype combinations (Zhang *et al.* 2004; Bennett *et al.* 2008; Wen *et al.* 2008).

It has been proved that the success of resynthesis greatly depends on the interactions between genotype and growth conditions, i.e., on days after pollination (DAP), the embryo rescue method, media composition, or temperature (Zhang *et al.* 2004; Bennett *et al.* 2008; Wen *et al.* 2008). Additionally, the choice of a suitable species as the female parental component is very important as well. Some reports referred *B. oleracea* as more successful female component in oilseed rape resynthesis than *B. rapa* (Ozminkowski and Jourdan 1994; Springer and Wojciechowski 2006; Wen *et al.* 2008). However, worse results have been reported in some crosses with the *B. oleracea* as a female; while the same but reciprocal combinations were considerably better (Wojciechowski 1985a; Lu *et al.* 2001; Malek *et al.* 2012). FitzJohn *et al.* (2007) reviewed the numbers of successful (S) and unsuccessful (U) attempts and identified *B. rapa* as significantly better female component (S 51,U 56) than *B. oleracea* (S 5, U 40).

2.2.4.2 Ovule Culture of Resynthesized *B. napus*

Ovule culture is preferred over direct excision of embryos, especially in small-seeded species and/or very young embryos, in order to prevent embryo damage (Reed *et al.* 2005). Interestingly, small superficial damage of the ovule at blade cut and pulling out of siliquae content may be beneficial as ovule perforations can increase water and nutrient uptake (Reed *et al.* 2005). The preparation process itself, prior to cultivation, is generally noted as time-consuming and difficult for small-seeded poly-ovulate species, as for example, when working with smaller and less robust *B. rapa* as the female parental component (Reed *et al.* 2005).

Nutritional requirements for optimal growth of a tissue *in vitro* may vary with the species. Even tissues from different parts of a plant may have different requirements for satisfactory growth (Murashige & Skoog 1962). As such, no single medium can be suggested as being entirely satisfactory for all types of plant and organs tissues and so for such a thing that develops into many physiological and morphological stages as an embryo, even as early as the one depending on the tissues surrounding it within an ovule.

When starting with a new material, it is essential to work out a medium that will fulfil most of the requirements for the specific period of development. Therefore, it may be enough to develop specific medium for one tissue only. However, for the culture of a proembryo to a plant there may be necessary different subcultures with different mediums and times. During the past 35 years, the need to culture diverse tissues and organs has led to the development of several recipes and most of the main basics can be found in Bhojwani and Razdan (1996). Furthermore, the embryogenic potential and the embryogenic response to concentrations of certain compounds in the medium as well as other requirements vary among genotypes. Embryo development is mainly influenced by phytohormones like auxin, gibberellins, abscisic acid and sometimes ethylene and also there have to be considered the nitrogen, sucrose and polyamines sources among other compounds.

Back to the fact that different phases of the embryo development require different culture conditions including the phase of embryo maturation (during which the embryo is prepared for germination), Bhojwani and Razdan (1996) described a very interesting situation: The embryogenic cells secrete certain proteins into the culture medium which not only help in maintaining the embryogenic potential by restricting cell elongation in the presence of auxin but also induce the appearance of small embryogenic cells in previously non-embryogenic cultures. This may induce other embryos than hybrids. On the other hand, too frequent subcultures may disrupt the embryo development pathways and produce only mere cell elongation by the interaction of auxins without any effect of these secreted proteins.

2.3 Diploidization of Resynthesized Hybrids

The next step after the creation of a new resynthesized amphihaploid or hybrid haploid is the chromosome doubling, the amphidiploid formation (Xu *et al.* 2012). This

general process, polyploidization, the multiplication of the whole chromosome complement has occurred frequently in vascular plants (Pecinka *et al.* 2011) and this phenomenon is not alien to *B. napus*. Furthermore, the exchange between homologous chromosomes is a very important factor for the origin of new allele combinations and phenotypic variation in resynthesized *Brassica napus* (Gaeta *et al.* 2007).

The efficient doubling protocol is even more necessary, as the reduced ploidy level is considered as quite stable (Grzebelus & Adamus 2004). A spontaneous doubling frequency of three new resynthesized crosses with 12, 15 and 21% was reported in Zhang *et al.* (2004). In parallel, regenerate plants were treated *in vivo*, dried two days and then plants were immerged into the colchicine solution at 85 and 170 mg l^{-1} for 20 and 30 h under glasshouse conditions. The best response to colchicine was observed when treated with 170 mg l^{-1} colchicine for up to 30 h and a doubling frequency of 52, 56 and 62%, respectively. Unfortunately, diploid chimeras are commonly observed only in *in vivo* colchicine treatments and diploidization rates and response to individual antimitotic compounds vary among genotypes (Klíma *et al.* 2008). On the other hand, the genotype response in many papers is difficult to evaluate and relevant statistical analysis is not always proved (Grzebelus & Adamus 2004).

The methods that involve the immersion of roots or whole plantlets in a colchicine solution require relatively large amounts of the expensive chemical while the rate of diploidization rarely exceeds 60% (Klíma *et al.* 2008). Despite these facts, colchicine has proven to be most useful to double the chromosome numbers of numerous crop species, including decorative flowers, medical and agricultural plants. This alkaloid, however, is very toxic to human beings and also shows undesirable mutagenic activity on plants (Grzebelus & Adamus 2004; Yemets & Blume 2008). Other application of colchicine was described at *in vitro* level for verified resynthesized hybrids with culture of ten days in a medium with 100 mg l^{-1} colchicine for chromosome doubling and then shifted back to medium without colchicine for rooting and subsequent transplantation in the field. Interestingly, the average doubling frequency was 75.3%. Smýkalová *et al.* (2006) rather submersed their plantlets in a solution of 0.5% (50 mg $l^{-1)}$ colchicine and 0.1% DMSO in a culture room at 20–22°C and 16 h photoperiod for 24 h. After colchicine treatment, the plantlets were placed onto sterile perlite saturated with half-strength MS medium for 2 weeks and regenerated plants were transferred to soil for further growth. The doubling

efficiency was on average 40.6% while Klíma *et al.* (2008) obtained 55.6%. Interestingly it still was less than the doubling efficiency of *in vitro* treated haploids from microspore cultures with colchicine, trifluralin or oryzalin.

2.3.1 Diploidization and Other Antimicrotubular Drugs

Microspore culture is a widespread technique in *Brassica* breeding described in chapter 2.5. Chromosome doubling is needed to obtain fertile plants and several studies with colchicines to accomplish it have been done. Beside colchicine, other antimitotic or microtubule depolymerizing compounds including dinitroaniline, oryzalin, trifluralin, and, more recently, phosphoric amide amiprophos-methyl were tested at various concentrations and for various periods of exposure (Champion *et al.* 1995; Geoffriau *et al.* 1997; Jakse *et al.* 2003). A review of the treatments including the kind of tissue treated and the most successful method of several papers is available in Dhooghe *et al.* (2011). These substances became a good alternative for colchicine since they have a much higher affinity to plant microtubules (Morejohn *et al.* 1987). Such chemicals can efficiently induce diploidization when used at the molar concentration up to 100–1000 times lower than colchicines. Therefore, the final cost of the procedure is reduced. Some papers have shown that *in vitro* diploidization of different explants may not be effective with the same methodologies, specific chemical and insufficient time and concentration (Dhooghe *et al.* 2011). Furthermore, in order to exclude mixoploids, it is often necessary to apply a combination of verification methods (see Chapter 2.6.1).

It is well known that high concentrations of these antimitotic agents can also produce other ploidy levels like mixoploids, triploids, tetraploids and octoploid or higher. Higher proportions of these ploidy fractions can occur in the presence of higher doses of these chemicals if treated longer time than suitable. For example, this was observed in 50 M trifluralin and oryzalin treated 24 h and 3 days, and there were no significant differences in haploid and diploid numbers between 1 and 3 days, but the proportion of higher ploidies was markedly increased by the longer exposure (Grzebelus & Adamus 2004).

2.4 Microspore Culture in *Brassica* Breeding

A very important part of the breeding programs are the methods to select desirable genotypes. Doubled haploids (DH) production technique of *in vitro* cultivated pollen grains (microspores) has been used routinely to accelerate the breeding process and to make the

breeding programs more effective. This method is also used for an early selection of specific characteristics at different stages of development of the microspore embryos (i.e., fatty acid content, disease resistances, cold tolerance, etc.; Klíma *et al.* 2004, 2008, Delourme *et al.* 2008, Takahira *et al.* 2011), stabilization of interspecific hybrids and transformations and molecular analyses (eg. Zhang *et al.* 2006, Formanova *et al.* 2006, Nath *et al.* 2009, Navabi *et al.* 2010). This method permits to obtain within 1-2 years a completely homozygous genotype, comparable in the required characteristics to a traditionally bred line, creating a combination of specific features that can be hardly reached by traditional breeding methods, such as auto-incompability (AI) and specific seed quality. DH system enables an efficient creation of breeding lines for both traditional varieties as well as composed hybrids and it is fully compatible with other biotechnological approaches, i.e. *in vitro* selection, often in combination with mutagenesis (Liu *et al.* 2005), DNA markers development and gene manipulations.

The microspore doubled haploid methodology is now employed in the majority of modern *B. napus* breeding programmes around the world as an alternative/supplement to conventional methods of homozygous line production (Klíma *et al.* 2008). The DH method reduces the time needed to develop and release new cultivars by approximately 2 to 4 years in comparison with conventional techniques. In the Czech Republic, the cultivation and breeding of oilseed rape have become increasingly dominated by hybrid varieties based on cytoplasmic male sterility (CMS) system. Microspore culture can aid in the selection of CMS restorer lines, and thus increase the efficiency of this programme. A biotechnological approach to the production of DH plants via culture of microspores may be successfully applied to affect a number of morphological (agronomic) qualitative traits contributing to both high yield and desirable seed composition. An important fact is that efficient selection of superior genotypes may be realized at an early stage of microspore culture *in vitro* by using one of the two cotyledons for fatty acid analysis while retaining the rest of the embryo (Möllers *et al.* 2000). Adamska *et al.* (2004) produced DH lines with a modified fatty acid composition related to substances of interest (i.e. fatty acids) heritable and environmental stability. Ferrie and Keller (2004) revealed that there were over fifty oilseed *Brassica* varieties in commercial production derived by a DH technique.

The breeding programme “Czech Winter Rape” is directed to the development of hybrid and line cultivars with improved seed yield and quality parameters such as modified

fatty acid composition, low glucosinolate content (15–20 μmol/g of seeds) as well as low erucic acid content (0.3–0.8%). In addition, improved resistance to biotic and abiotic stresses and resistance to frost, lodging and fungal diseases (*Phoma lingam*, *Sclerotinia sclerotiorum*) are other significant limiting factors for high yield of oilseed rape (Odstrcilová and Plachká 2005). Thus, the creation of high quality F_1 hybrids used as donor plants for biotechnological approaches is a crucial point which could significantly help conventional methods to produce new oilseed rape varieties.

In the eighties, mainly spring rape cultivars such as Topas served as models for the optimization of the protocol (Coventry *et al.* 1988). The method was adopted and modified for Czech winter rape cultivars and breeding lines by Vyvadilová and Zelenková (1992) and later applied in the Czech programmes of *Brassica* spp. breeding (Vyvadilová *et al.* 1998a,b). Despite the fact that microspore-derived doubled haploid production protocols are well elaborated (and exhibit high efficiency) in model genotypes (i.e. Topas), they cannot usually be simply used for a broad spectrum of breeding materials/lines (Ferrie *et al.* 1995) and should be modified/optimized individually in order to obtain satisfactory efficiency for practical breeding purposes; i.e. *B. napus* and cruciferous vegetables.

Regeneration of doubled haploid plants from microspores can a useful tool for acceleration of homozygous line production in breeding programmes of other *Brassica* crops. The successful haploid embryo production in microspore cultures was reported in most *Brassica*ceae species (Lichter 1989; Duijs *et al.* 1992). *Brassica oleracea* L. includes several remarkably different morphological forms. Such forms also differ in their ability to produce haploid regenerants (Rudolf *et al.* 1999). The problem of practical application of the microspore culture technique is a very low embryo yield in many of core crop genotypes (Carlos & Dias 1999). Sufficient production of microspore derived plants in a wide range of genotypes is a prerequisite for the use of a doubled haploid system for rapid introduction of specific traits in *Brassica* vegetable breeding. Improvement of the microspore culture technique and investigation of factors affecting embryogenesis in a microspore culture of selected *Brassica oleracea* vegetables were studied but statistical analysis did not prove a significant effect of any level of individual factors (Vyvadilová *et al.* 1998 a,b). However, the main factors affecting microspore embryogenesis are understood as genotype specificity (Rudolf *et al.* 1999) and developmental stage of microspores (Möllers *et al.* 1994).

Development of DH lines by means of *in vitro* microspore culture has been increasingly used for homozygous line production in breeding programmes of *Brassica* crops due to a possibility of significant time reduction of cultivar development. Whereas this method is routinely used in oilseed rape breeding, it is still difficult to apply microspore culture techniques in practical breeding of some vegetable *Brassica*s. The main difficulties are the very low embryo yield and insufficient regenerative ability of microspore-derived plants in many of *Brassica* genotypes (Carlos & Dias 1999; Rudolf *et al.* 1999). Abnormal embryos occur very often and need to be subcultured many times to induce normal shoots (Kuginuki *et al.* 1999). Direct and rapid plant regeneration is very important for eliminating cytogenetic abnormalities and improving the efficiency of DH system. Other studies aimed to increase the efficiency of the microspore culture technique, especially by investigating factors affecting pollen embryogenesis and testing the embryogenic responsibility in a broad range of genotypes from *Brassica* collection and some breeding materials (Vyvadilová *et al.* 1998 a, b). However, the microspore derived embryo regeneration and frequency of fertile doubled haploids are still unsatisfactory. Thus, the optimization of the above mentioned techniques is still required.

2.5 Chromosome Doubling in Microspore Cultures

Various chromosome doubling techniques of microspore regenerants by colchicine treatment have been investigated. However, the methods that involve the immersion of roots or whole plantlets in a colchicine solution are laborious, time consuming and require relatively large amounts of an expensive chemical. These procedures may result in ploidy chimeras, poor seed set and a usually bellow 60% diploidization rate (Klíma *et al.* 2008). Microspore cultures can have around 10–20% spontaneous diploidization, but the rate of the diploidization as well as the embryogenesis can be significantly increased if colchicine is applied directly on the medium (Klíma *et al.* 2008). Several microtubule depolymerising herbicides were also proved to be efficient for *in vitro* chromosome doubling of microspores. Zhao & Simmonds (1995) tested trifluralin and Hansen & Andersen (1996) trifluralin, oryzalin and amiprophosmethyl in the spring rapeseed cultivar Topas. They achieved the mean rate of diploidization from 60% to 65%, which is comparable with the application of colchicine. Klíma *et al.* (2008) obtained even higher diploidization rate (85.7%) in winter rapeseed when they used trifluralin as an antimitotic agent. In addition,

herbicides have an advantage because of their lower toxicity than colchicine and because lower concentrations are needed for the treatment.

2.6 Verification Methods

In addition to the embryo production, reliable and early verification of hybridization seems to be equally important, as some authors have reported the non-negligible incidence of female components among regenerants derived via ovule culture or embryo rescue technique (Wen *et al.* 2008; Sosnowska *et al.* 2010). Verification at an early stage has a key role to save space, time and costs.

2.6.1 Flow Cytometry vs Other Methods

Concerning the morphological assessment of hybrid origin, contrasting phenotypes as the male component, should result in favourable, combinations. However, limitations of this assessment exist especially when the genotypes are similar (closely related), especially at early stages, indicating that reliable identification of hybrid plants at very early developmental stages is clearly needed (Carloni *et al.* 2014).

If hybrids are not readily distinguishable using morphological markers, such verifications methods can be confirmed in combination with flow cytometry as it can represent a valuable tool in identifying hybrids (Eeckhaut *et al.* 2005; Nakamura *et al.* 2005; Benavente *et al.* 2008). Thus, it is convenient to reliably identify hybrid plants in very early developmental stages whereas cases of self-pollinated contamination would be eliminated avoiding vain cultivation of excessive material. Although cytological analyses have been used for many years to confirm the hybrid nature, this well-known technique was evaluated as time consuming and disadvantageous one compared to FCM (Takahira *et al.* 2011). Therefore, using FCM for early detection of hybrids will be even more desirable in cases of using low contrast parents where morphological evaluations may not be applicable.

The use of flow cytometric analyses for RS oilseed rape hybrid verification is well-known (Heath & Earle 1995, 1997) and provides the advantage of verification at an earlier stage of plant development where the process becomes precise, fast and simple thanks to different genomic values of the parental species (Eeckhaut *et al.* 2005; Nakamura *et al.* 2005; Benavente *et al.* 2008). Therefore, this method becomes superior to karyology

(Scarano *et al.* 2003) although possible chromosome elimination or asymmetrical hybridisation is rather studied by exhaustive chromosome and cytological studies (Heath & Earle 1995). Furthermore, despite other methods, the accuracy of molecular and biochemical methods such as molecular markers or biochemical assessments increases with complexity of the process and the number of markers (Budak *et al.* 2004; Benavente *et al.* 2008) while FCM supplies it by a quick ploidy analysis (Navrátilová *et al.* 2004). These molecular markers, namely restriction fragment length polymorphism (RFLP), simple sequence repeat (SSR), amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA (RAPD) as well as isozyme markers are instead widely used to investigate the level of genetic diversity present in *B. napus* germplasm (Rahman 2013).

In previously published studies, flow cytometry has been used for RS oilseed rape hybrid verification (Ozminkowski & Jourdan 1994; Heath & Earle 1995; 1997; Sosnowska *et al.* 2010). It has been stated that divergence in genome size between the parental components should reach at least 6–8% in order to reliably distinguish hybrids via flow cytometry (Suda *et al.* 2007). Concerning *B. rapa* and *B. oleracea*, Johnston *et al.* (2005) observed the average difference about 31%. As a result, the number of analyses, similarly as described by Schranz *et al.* (2005) and Krejcíkova *et al.* (2013), may be reduced. Thus, flow cytometry with the use of bulked samples can contribute greatly to the reduction of the time and costs without compromising reliability of the method and prove to be an excellent tool for detection of hybrid regenerants at early stages of *in vitro* regeneration.

2.7 Seed (Oil) Quality in Rapeseed

Rapeseed oil, as it was previously stated, is used for different purposes. Generally, two kinds of rapes are breed; LEAR, Low Erucic Acid Rapeseed, ie, canola (or double “00” for low erucic and glucosinolate content) for oil comsuption and seed meal or HEAR, High Erucic Acid Rapeseed for industrial purposes. The most important quality parameters of rapeseed therefore are high oil content and low glucosinolate content, but also the specific composition of fatty acids profile in the oil, with erucic acid content in the first place. While in HEAR high erucic acid content is desired (> 43%), low (< 5%) concentration of such acid is required in LEAR.

Other traced fatty acids are: oleic linoleic and linolenic acids (around 63.4 g, 17.5 g and 6.9 g respectively in 100g of rapeseed oil). Palmitic acid (around 4.3 g in 100g of rapeseed oil) is also found in a significant amount and others like stearic, eicosenoic, eicosedinoic, behenic, docosadienoic, docosahexaenoic and lignoceric acid among others complete in lesser amounts the fatty acid profile. The fatty acid composition can be very precisely analysed by Gas chromatography (GS) (chapter 5.8.1), and less precisely by the use of defined standards by Near Infrared Spectroscopy (NIRS) (chapter 5.8.2). NIRS on the other hand can also analyse simultaneously protein, glucosinolate, nitrogen compounds, oil, and humidity content, reviewed in Gómez-Campo (1999).

The properties of non-saturated acids are very important as they have health and economical importance. They are not only an energetic source for the human organism, but they are mainly part of the phospholipids of cell membrane (Suchý *et al.* 2008). High oleic acid content in rapeseed oil is also desired because it lowers the levels of "bad" LDL cholesterol by replacing saturated fatty acids. Even more important for its use is that it is stable at high temperatures, so that it is suitable for frying. Linoleic acid has a similar effect on cholesterol and it is one of the essential fatty acids which the human body needs for the construction of cellular membranes and the production of hormones (prostaglandines, tromboxanes, leukotrienes). However, our body is not able to create it itself and we need to take it from food. On the other hand, linoleic acid is not as stable as oleic acid and a higher content would make canola oil less suitable for frying. This fatty acid is an omega 6 and linolenic fatty acid is classified as an omega 3. Linolenic acid is also very important as its deficiency in human comsuption has been associated with the occurrence of vascular and heart diseases. In paralel to fatty acid composition of rapeseed oil, low glucosinolate content (< 15–20 μmol/g of seeds) is desired for seed meal (Szala *et al.* 2016). Seed yield together with the seed quality parameters in one genotype can vary according the growing conditions. Specifically, oil content and Erucic acid percentage seems to be significantly affected by climate conditions (Suchý *et al.* 2008).

Regarding to seed quality and breeding, the direct use of RS oilseed in breeding programmes is limited by their quality: the seeds have high glucosinolate and erucic acid content originating from *B. oleracea* and *B. rapa* (Girke *et al.* 2012; Jesske *et al.* 2013; Rahman 2013). It was seen in Jesske *et al.* (2013) that the heritability of yield, oil content, protein content, erucic acid content and glucosinolate content were high while winter

hardiness and lodging had low heritability. However, for hybrid breeding, the effect of heterosis obtained by crosses of genetically diverse genotypes is very important to improve the seed yield. The cross of resynthesized *B. napus* and rapeseed cultivars significantly increase the genetic diversity, which is often correlated with the effect of heterosis (Wu *et al.* 2014). Furthermore, as stated by Wu *et al.* (2014), a high rate of allele mutations along with allele introgression and the heterosis effect in RS *B. napus* and its F_1 hybrids might collaborate with higher yield of the derived hybrids. RS lines are promising for obtaining secondary genepools with new genetic diversity in which we can include new allelic combinations and mutations derived from the crosses with RS lines (Wu *et al.* 2014). This new combinations in turn can result in interactions that contribute to new sources of changed oil profiles (Lu *et al.* 2001). Interestingly, resynthesized rapeseed genepool is not suitable for direct breeding of oilseed rape because of the undesirable agronomic traits derived from one or both progenitors. However, the seed quality issues can be overcome by crossing with quality donors and the use of double haploids through microspore cultures (Szala *et al.* 2016).

3 HYPOTHESIS

1. Resynthesized oilseed rape can be obtained by crossing selected genotypes of *B. rapa* and *B. oleracea*, with an optimized embryo rescue protocol based on scientific research and derived on modern winter *B. napus in vitro* cultivation.

2. It can be made a suitable protocol for a wide range of crosses, and therefore, usable in breeding programs. Interspecific hybrid production rate will also depend on the physiological and genomic compatibility of the starting components.

3. Winter oilseed rape type, requiring vernalisation for the transition to the generative state can be resynthesized by crossing selected genotypes of winter rape and kale. The combination of a spring turnip rape cultivar with a cabbage will have more or less a vernalisation need.

4. Sexual crosses can give rise to false hybrid embryos originated only from *B. rapa*. Polyploidization of RS plantlets can produce fertile RS rapes but also chimeric plants, with different ploidies and problems with fertility.

5. There is a correlation between the seed quality parameters of one or both starting parents (*B. rapa*, *B. oleracea*) and levels of the seed quality parameters of resynthesized rapeseed created from these parents.

6. Resynthesized rapes from selected genotypes of *B. rapa* and *B. oleracea* genotypes can be obtained with a specific fatty acids oil content or other unique features absent in the available range of varieties of rapeseed.

7. Based on the embryogenicity of a donor quality rape, fertile RS material can be crossed with elite rapeseed cultivars in order to stablish dihaploid lines from microspore cultures and therefore, to select lines with specific fatty acids composition in the oil and glucosinolate content in the seed of the resulting interspecific cross.

4 OBJECTIVES

The main objective of this thesis was to optimize and apply selected biotechnological methods in the resynthesis of oilseed rape (*B. napus*) from its baseline species (*B. oleracea*, CC and *B. rapa*, AA) with different selected genotypes and to subsequently regenerate oilseed rape genotypes with desired characteristics.

The partial aims were:

Ad 1) To optimize the embryo rescue protocol in *B. napus* and further optimize it with newly resynthesized material (material difficult to obtain). To implement a simple and efficient embryo rescue technique through ovule culture.

Ad 2) To obtain embryos from a wider number of accessions in the intespecific cross of *B. rapa* and *B. oleracea* and analyse the crossability parameters of oilseed rape resynthesis and regeneration of obtained hybrids. To evaluate the hybrid production rate of the crossed genotype combinations.

Ad 3) To produce resynthesized winter oilseed rape type, requiring vernalisation for the transition to the generative state from crossing selected genotypes of winter rape and kale.

Ad 4) To verify the hybrid nature of the hybrid material by means of morphological assessments, flow cytometry and other techniques. To apply polyploidization agents and to obtain diploid plants with evaluated polyploidization rate and fertility.

Ad 5) To evaluate and select traits in the starting parents and their hybrids by their properties; spring/winter type, fertility and seed quality.

Ad 6) To determine new features or properties, i.e. specific fatty acid profiles among the RS lines, which could contribute to broaden the rapeseed genepool.

Ad 7) To test the embryogenic potential and to obtain seed of self-pollination and/or seeds of hybrids with an elite line of *B. napus.*

5 PLANT MATERIAL AND METHODS

5.1 Plant Materials

5.1.1 Conventional Oilseed Rape

Accessions of winter oilseed rape; *Brassica napus* L. subsp. *napus* f. *biennis* THELL: Benefit, Cadeli, Californium, Navajo and Viking were used for experiments regarding optimization of embryo rescue and ovule culture by means of different media, days after pollination (DAP), morphology and time of culture. The materiál was grown, self- pollinated in bud, labeled and bagged acording to standard protocols. It was aimed to develop a procedure which will produce, at least, some embryos at the shortest possible number of days after siliquae harvest (the number of cultivations with success were of interested as they meant the time in which it is possible to sucessfuly obtain embryos in early embryo rescue) in conventional rapeseed cultivars.

5.1.2 Plant Components for Resynthesis

Nine accessions of winter turnip rape (*Brassica rapa* L. ssp. *oleifera* (DC.) Metzg. f. *Biennis):* Arktus, Brachina, Bulharska, Grubers Winterrübsen, Izumrudnaja K 193, Svalöfs Duro, Ludowy, Slezsky Krajový and Rapido, three accessions of spring turnip rape (*Brassica rapa* L. ssp. *oleifera* (DC.) Metzg. f. *praecox):* Jumbuck, Ante, Evissa, and one spring white turnip rape (*Brassica rapa* L. ssp. *rapa* (DC.) Metzg.*)* breeding material: V17 (Billa) were used as female components (turnip rape, 2*n* = 20, AA) for the resynthesis experiments. Two accessions of cabbage (*Brassica oleracea* L. convar. *capitata* L.): Vysocké AIK, Zakamenné and seven accessions of winter curly kale (*Brassica oleracea* L. convar. *acephala* (DC.) Alef. var. *sabellica* L.): Frosty, Kadeřávek Zelený, Kapral, Scarlet, Pentlang Brig, Nero di Toscana and Vates were used for experiments as male components (Cabbage and Curly kale, 2*n* = 18, CC). The genotypes were selected by contrasting morphological characteristics (cabbage and curly kale) or agronomical traits (turnip rape). Accessions of *B. rapa* were obtained from OSEVA Development and Research, Ltd., Oilseed Research Institute at Opava, Czech Republic except for Vodnice Billa which was obtained from the supermarket chain Billa. Accessions of *B. oleracea* were obtained from the Crop Research Institute Prague (both cabbages), Centre for Applied Research of Vegetables and Special Crops at Olomouc (Curly kales except two) and from MORAVOSEED, Ltd., Czech Republic (Kapral and Scarlet).

5.1.2.1 Plant Materials of Experiment 1

In order to obtain winter type RS rape, twenty-four one-sided crosses between winter turnip rapes and winter curly kales were used. Six accessions of winter *Brassica rapa* L. ssp. *oleifera* (DC.) Metzg. f. *biennis* (winter turnip rape, 2*n* = 20, AA) Arktus, Brachina, Bulharska, Grubers Winterrübsen, Izumrudnaja K 193, and Svalöfs Duro; and four accessions of winter *Brassica oleracea* L. convar. *acephala* (DC.) Alef. var. *sabellica* L. (winter curly kale, 2*n* = 18, CC) Frosty, Kadeřávek Zelený, Kapral, and Scarlet (see abbreviations in Table 2). The genotypes were selected due to their contrasting morphological characteristics (curly kale) or agronomical traits (turnip rape). Accessions of *B. rapa* were obtained from OSEVA Development and Research, Ltd., Oilseed Research Institute at Opava, Czech Republic. Two accessions of *B. oleracea* were obtained from the Crop Research Institute, Centre for Applied Research of Vegetables and Special Crops at Olomouc, and two were from MORAVOSEED, Ltd., Czech Republic (Table 2).

Table 2 *Brassica* accessions used for resyntheses.

Accession (Cultivar)	Abbr.	Country of Origin	Seed provider (Item No.)	Selected traits (EVIGEZ 2013)*
B. rapa (female parent)				
Arktus	AR	Germany	15*O*0300001	Taller plants, longer siliquae
Brachina	BR	Poland	15*O*0300034	Earlier flowering, higher winter hardiness
Bulharska	BU	*unknown*	15*O*0300003	Shorter plants, higher oiliness
Grubers Winterrübsen	GW	Germany	15*O*0300004	Higher oiliness
Izumrudnaja K193	IZ	USSR	15*O*0300027	Reduced GSL and erucic acid content
Svalöfs Duro	SV	Sweden	15*O*0300019	Lodging resistance
B. oleracea (male parent)				
Frosty	FR	Netherlands	09*H*1900158	Dwarf, compact habit
Kaderavek Zelený	KZ	Czech Rep.	09*H*1900139	Taller plants
Kapral	KA	Czech Rep.	*M*	Dwarf habit
Scarlet	SC	Czech Rep.	*M*	Taller plants, dark purple leaves

*Descriptors available only for *B. rapa*; Seed providers: O – Oilseed Research Institute; H – Centre for Applied Research of Vegetables and Special Crops; M – MORAVOSEED Ltd

5.1.2.2 Plant Materials of Experiment 2

In order to obtain a wide range of genetic germplasm accessions of winter turnip rape (*B. rapa*. ssp. *oleifera* f. *biennis)* Arktus, Brachina, Bulharska, Grubers Winterrübsen, Izumrudnaja K 193, Svalöfs Duro, Ludowy, Slezsky Krajový and Rapido, and accessions

of spring turnip rape *(B. rapa*. ssp. *oleifera* f. *praecox)* Jumbuck, Ante-12, Ante-27, Evissa, and one breeding material of spring turnip rape (*B. rapa* ssp. *rapa*) V17, were used as female components, while accessions of cabbage *(B. oleracea* convar. *capitata*) Vysocké AIK, Zakamenné and winter curly kale (*B. oleracea* convar. *acephala* var. *sabellica*) Frosty, Kadeřávek Zelený, Kapral Scarlet, Pentland Brig, Nero di Toscana and Vates were used for experiments as male components. Genotypes were selected by contrasting morphological (cabbage and curly kale) or agronomical traits (turnip rape).

5.1.2.3 Plant Materials of Experiment 3

In order to focus on the consequently regeneration of fertile plants, able to set seed via conchicine treatment, three accessions of spring oilseed turnip rape (*Brassica rapa* L. ssp. *oleifera* f. *praecox*, 2*n* = 20, AA) Ante, Evissa, one breeding matrial of white turnip (*B. rapa* ssp. *rapa*) V17 and four accessions of winter oilseed turnip rape (*Brassica rapa* L. ssp. *oleifera* f., *biennis*, 2*n* = 20, AA), Brachina, Izumrudnaja K 193, Ludowy and Svalöfs Duro were used as the female component. Two accessions of cabbage (B. oleracea convar. capitata) Vysocké AIK and Zakamenné. These spring and winter turnip rapes and cabagges were used for the resynthesis based on contrasting characteristics (Table 3).

Table 3 Genotypes properties and characteristics, used for oilseed resynthesis

Material (variety)	**Characteristics and properties**
Ante	High trichomes density, high resistance to lodging
Brachina	Early flowering, high resistance to winter kill
Evissa	High resistance to lodging
Izumrudnaja K193	Low glucosinolate and erucic acid content
Jumbuck	Lighter color of testa
Ludowy	Resistance to winter kill and dense inflorescence
Svalöfs Duro	High resistance to lodging
V17	Compact habit
Vysocké AIK	Conical loaf, crimson cabbagehead color
Zakamenné	Green cabbagehead color

5.1.2.4 Plant Materials of Experiment 4

Two resynthesized combinations from spring turnip rape genotypes “Ante” and “Jumbuck”, and the cabbabe “Vysocké” were self-polinized and crossed with productive winter oilseeds genotypes as quality donors to obtain seeds (Cadeli with Ante × Vysocké and Ladoga with Jumbuck × Vysocké). Seeds qualities were analysed by NIRS (Chapter

5.8) and compared. This experiment was concepted for the hypothesis that desired properties of resynthesized rape can be transferred to conventional crossbreeding varieties or breeding materials.

5.1.2.5 Plant Materials of Experiment 5

In order to determine the fatty acids (FAs) composition in oil of resynthesized (RS) rapeseeds and find a relationship between the concentrations of individual FAs, and between FA profiles of parental components and F_1 crosses with the conventional variety Ladoga, female components of two spring genotypes of turnip rape Jumbuck and Ante with high oil quality breeding material turnip rape V17 and two turnip rape winter genotypes of turnip rape Izumrudnaja a Brachina were used. The used male components were Dihaploid line of cabbage *(B. oleracea* convar. *capitata*) Vysocké AIK and three genotypes of curly kale *(B. oleracea* var. *sabellica*) Scarlet, Vates a Kapral. Seven RS materials taken from the optimized procedure (Hilgert *et al.* 2015a) were selfed in bud and crossed with conventional quality donor of winter oilseed rape, Ladoga at the Crop Research Institute (Table 8). The seed quality of RS materials (including their parental components) and their crosses with elite varieties of winter oilseed rape were tested by comparing seed quality parameters through gas chromatography.

5.1.2.6 Plant Materials of Experiment 6

The aim of this experiment was to study the seed set of RS lines crossed with Ladoga, determine if the crossed combinations would have viable seed set and be either autocompatible or autoincompatible and determine their embryogenic potential and the regeneration rate of microspore cultures.

The used female components were six RS materials obtained in or with the same procedure as in Hilgert *et al.* (2015b): Arktus × Pentland Brig, Bulharská × Kadeřávek Zelený, Izumrudnaja k 193 × Kapral, Grubers Winterrübsen × Frosty, Grubers Winterrübsen × Scarlet and Svalöfs Duro × Kadeřávek Zelený. The used male component was the winter oilseed rape Ladoga (*B. napus* convar. *napus*), as a quality donor and embryogenic donor for microspore cultures. The materials crossed were named after the cross according to table 4. Twelve plant from each cross were vernalisated and the best six plant of each were used for experiments.

Table 4 Material used in Experiment 6

RS components	Donor	Name of the cross
Arktus × Pentland Brig	Ladoga	Arkpet × L
Bulharská × Kadeřávek Zelený	Ladoga	Buzel × L
Izumrudnaja k 193 × Kapral	Ladoga	Izuka × L
Grubers Winterrübsen × Frosty	Ladoga	Grusty × L
Grubers Winterrübsen × Scarlet	Ladoga	Grusca × L
Svalöfs Duro × Kadeřávek Zelený	Ladoga	Svazel × L

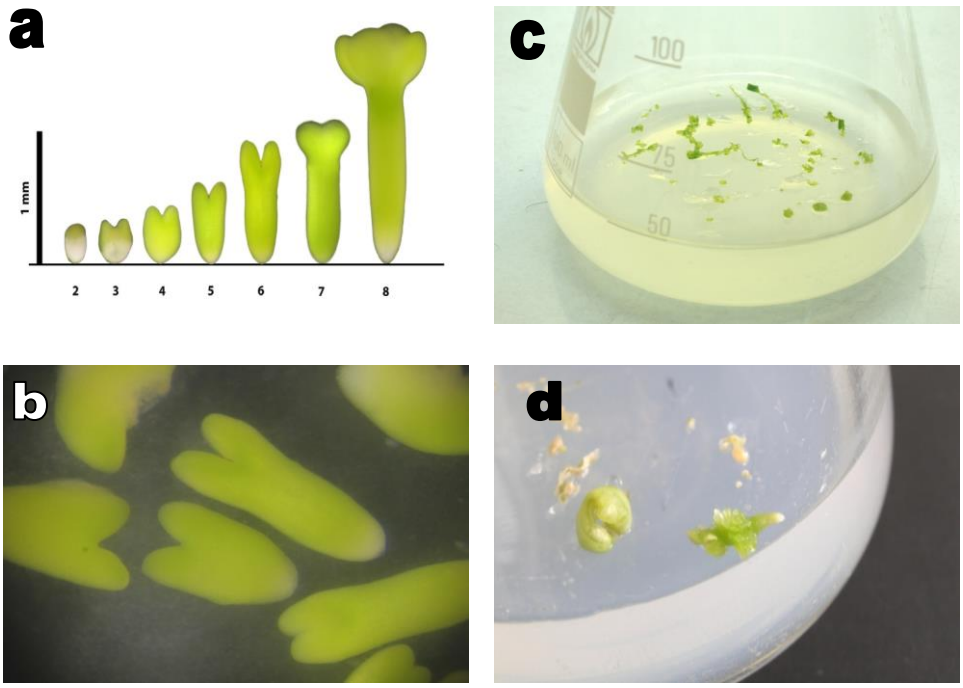
5.2 Optimization via Direct Cultivation of Modern *B. napus* Varieties

The first year the work was aimed to work on conventional *B. napus* buds in order to easy obtain large quantity of siliquae from which embryos could be cultured *in vitro* similarly as for microspore cultures on different media (Klíma *et al.* 2004). Optimized cultivation process was assessed from embryo and ovule culture (for difference see Fig. 2) together with different harvest DAP for the recognition of the earliest possible period of time after pollination matched with studied media, which all together, would allow to avoid incompatibility barriers of the future resynthesized material and best culture conditions. Flower buds of *B. napus* which had dehisced in 24–72 h were emasculated and immediately pollinated with fresh *B. napus* pollen, labelled and bagged using conventional methods to avoid undesirable pollination. This step allowed the study of siliquae development and siliquae were evaluated mainly in width and length before harvested after 28, 21, 16, 14, 10, 7 and 4 days. One pollination cycle for each genotype at 28, 21 and 14 days and three pollination cycles for 4, 7 and 10 days with 20 buds in each replication were made to register the embryos development. Ovules of 4, 7, 10 and 14 days were taken out of the siliquae and placed in equal quantities on the top of three modified media used by Klíma *et al.* (2004): Differentiation, Regeneration and Liquid NLN-13 medium with 13% sucrose (Lichter 1982). Cultivation procedure was according to Bennet *et al.* (2008) for ovule culture in liquid medium. NLN-13 medium was filter-sterilized (0.22 μm filter, Millipore) under sterile conditions in air laminar flow; solid media were sterilized by autoclaving (121°C, 15 min). Trial experiments were also tested directly on MS medium (Murashige & Skoog 1962). Embryos were counted at the 28th day of culture and were not further cultured.

5.3 Crossing and *in vitro* Cultivation

Donor plants were grown in the soil in a greenhouse for 4 weeks. Then, the plants with 3 to 4 developed leaves were transferred to a vernalisation chamber (except for the spring types) at 4 °C, and 12 h photoperiod (irradiance 20.4 μmol m^{-2} s^{-1}) for subsequent 7-8 weeks. Spring types and vernalized winter type parental components were grown under a controlled environment in a growth chamber (light intensity 84 μmol m^{-2} s^{-1}, 17/15 °C day/night, and photoperiod 16/8 h) to the stage of flowering plants.

Fig. 2 Embryo Rescue vs Ovule Culture



Embryo Rescue in liquid medium: (a) stadia of embryo development. (b) Early and late torpedo stadium. Ovule Culture in solid differentiation medium: (c) fresh ovules and other gren parts of the silique. (d) Embryo and 30-days-old-parts of the siliques.

Fig. 3 *B. rapa* pollinised buds with pollen of *B. oleracea*

Buds pollinised 11 days after emasculation.

5.3.1 Interspecific Crosses and *in vitro* Cultivation

Flower buds of *B. rapa* which would have dehisced 24–72 h later were emasculated and immediately pollinated with fresh *B. oleracea* pollen, labelled, and bagged using conventional methods to avoid undesirable pollination (Fig. 3). In total, forty two different crosses were made between selected *B. rapa* (female component) and *B. oleracea* (male component) accessions in three pollination cycles, with 30 buds in each replication in experiment 1, and with 20 buds in each replication in experiments 2 and 3. In order to determine the pollination efficiency, well-developed siliquae were harvested 11 days after pollination (DAP) and counted. The siliquae were surface-sterilized for 2 min in 70% (v/v) ethanol, followed by 10 min in 1.5% (v/v) sodium hypochlorite solution, and then washed three times for 5 min in sterile deionized water. Prior to ovule isolation, the siliquae were kept in cold sterile water for up to 2 h. The siliquae were first despoiled of the end part containing no ovules, and then carefully slit lengthwise with a surgical blade (preferably only at one junction between the valves to pull off their contents from the very first ovule to the cut end) (Fig. 4*a*). All ovules and/or septum with ovules were taken out of the siliquae and cultured (Fig. 4*b*) on the differentiation (D) medium (Klíma *et al.* 2004), in 50

ml Erlenmeyer flasks, supplemented with: 0.8% (w/v) agar, 2% (w/v) sucrose (both high-grade, Sigma-Aldrich, St. Louis, MO, USA), 0.2 mg l^{-1} BA, 0.2 mg l^{-1} IAA, at pH = 5.8. Cultures (ovules from approx. five siliquae per flask) were maintained at 25°C, with a photoperiod of 16/8 h, and a light intensity of 300 μmol m^{-2} s^{-1}. Embryos were counted on the 35th day of culture, when they had reached a length of 2–4 mm (Fig. 4*c*-*e*), and then subcultured on fresh D medium for 10–20 days to accelerate regeneration (Fig. 4*f*-*i*). Further cultivation and regeneration of whole plants (Fig 4*j*, *k*) was carried out according to Klíma *et al.* (2004), except for the removal of cotyledons.

5.3.2 Micropropagation

The method of creating *in vitro* cultures and cloning the plant material of all species investigated was specific to each type according to selected literature (Klíma *et al.* 2004, 2008). Micropropagation was performed in well-developed plantlets, where they were multiplied from nodal segments and subcultured every three weeks or according to rooting and growth of the cultured plantlets onto MS medium (Murashige & Skoog 1962) under cultivation temperature 20/15°C.

5.4 *In vivo* Diploidization

After the hybrid nature of the lines with giving rise to well regenerated plants was confirmed, at least one line per combination was aimed to clone into 20 to 30 plants and best ten to twenty rooted plants of them were submersed in a solution of 0.5% (50 mg $l^{-1)}$ colchicine and 0.1% DMSO in a culture room at 20–22°C and 16 h photoperiod for 24 h. Then they were washed with tap water at room temperature. Then, the plants were transferred to sterilized perlite and they were maintained at 25 °C, photoperiod 16/8 h and a light intensity of 300 μmol m^{-2} s^{-1} for four weeks in boxes with transparent covers to retain humidity. The covers were gradually loosened for acclimation to normal air humidity. Recovered plants were transferred in the soil and further grown in a greenhouse up to the stage of at least 4 leaves, when they were subjected to vernalisation if necessary in a vernalisation room (6°C, 16 h photoperiod, irradiance 56 μmol m^{-2} s^{-1}) for 6 weeks (Smýkalová *et al.* 2006; Klíma *et al.* 2008).

5.5 Statistical Analyses on Embryo Production

Pollinated buds, which later led to lost siliquae during manipulation, or to contaminated cultures and embryos as well as calli, were excluded from the counting and statistical analyses. Pollination efficiency was computed as the ratio between the number of siliquae, with at least one ovule developed, and the number of flower buds pollinated. The hybrid siliquae ratio (HSR) was the mean number of embryos per one cultured siliquae, and the hybrid production ratio (HPR) was the mean number of embryos obtained from one cross-pollinated bud. The percentage of the data subjected to analysis of variance was modified by means of angular transformation in order to follow a normal distribution of errors (Gómez & Gómez 1984). Homogeneity of the variance was accessed via Levene's test, followed by tests of variance (parametric or non-parametric, according to the Levene's test), multiple comparisons of means, Spearman correlation, and simple linear regression computed in statistical software (STATISTICA 10.0, StatSoft Inc., UK).

5.6 Flow Cytometric Analyses

The relative DNA content of all parental components, as well as hybrids, was assessed by flow cytometric analysis. Ten randomly chosen plants from all parental cultivars were analysed three times on different days. Up to five regenerants, originating from different embryos of identical crosses, were processed together in two replications. The two-step method for FCM analyses was used, according to Dolezel *et al.* (2007). Approximately 1 cm^2 from each plant, and an appropriate amount of internal standard (*Zea mays*, cv. CE-777, 2C = 5.43 pg; Lysak & Dolezel 1998), was chopped up with a razor blade in 0.5 ml of Otto I buffer (0.1 M citric acid, 0.5% (v/v) Tween 20). The suspension was filtered through a 42 μm nylon mesh. After ten min incubation at room temperature, 1 ml of Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12\ H_2O$), 4 μg ml^{-1} 4',6-diamidino-2-phenylindole and 2 μl ml^{-1} β-mercaptoethanol was added. The relative fluorescence intensity of at least 3,000 nuclei was measured using a CyFlow Space flow cytometer (Partec GmbH, Münster, Germany). The data were analyzed using FloMax software, version 2.4d (Partec, GmbH, Münster, Germany).

For each analysed sample, the DNA ratios were counted by dividing the mean of the G1 peak of the studied plant by the mean of the G1 peak of the internal standard.

Statistical analysis of the data obtained from FCM analyses was performed by analysis of variance (ANOVA), and the significantly different means were identified by using the Tukey's HSD test (P = 0.05) (STATISTICA 10.0, StatSoft Inc., UK).

5.7 Leaf Morphology and Chromosome Counts

Morphology was assessed at the stage of at least five true leaves by the comparison of selected characteristics (preferably leaf shape and/or colour) of regenerants and the female parental components. The obtained plants were also surveyed for possible morphological markers, inherited from the respective male component.

Excised root tips were pretreated with a saturated solution of p-dichlorobenzene for 2 h. Pretreated samples were fixed in an ethanol-acetic acid (glacial) 3:1 (v/v) mixture at 5 °C overnight. After maceration in 1:1 (v/v) hydrochloric acid:ethanol for 20 s, and subsequent rinsing in deionized water for 15 min, the root tip cuttings were squashed in saturated lacto-propionic orceine solution. The slides were evaluated with a Carl-Zeiss Jena NU microscope equipped with an Olympus Camedia C-2000 Z camera. The root-tip chromosome counts were performed on ten randomly selected regenerants, and compared with both parental components.

5.8 Oil Content in Seed and Fatty Acid Composition

5.8.1 Gas Chromatography (GC)

The assessment of fatty acids methyl esters were made by means of gas chromatography analysis, optimized for minimal sample weight (Endlová *et al.* 2013, 2014) and the fatty acid content was expressed as a percentage of individual acids. Samples of seeds prepared at Crop Research Institute Prague were sent to the laboratories at OSEVA Development and Research Ltd, Opava, where they were pulverized, transferred to a glass vial and dried at 40 °C for 48 hours. An isopropanole/isooctane solution, mixed in the ratio 1:9 (v/v) was added to each vial and thoroughly homogenized. Shattered samples were tempered at 55 °C and dried by nitrogen. The samples were resuspended in the mixture of 50 µl of sodium methanolate and 150 µl of isooctane. Homogenized suspensions were transferred into micro test tube and both phases were separated - only upper, i.e. isooctane phase was used for analysis. Composition of fatty

acids was determined by gas chromatography with subsequent detection (GC / FID). A gas chromatograph type Master GC (DANI Instruments SpA) with a flame ionization detector was used to identify and determine the ratio of the substances studied. Subsequent analyses were carried out according to standard protocols.

5.8.2 Near Infrared Spectroscopy (NIRS)

Seed samples were analyzed for oil content, fatty acids composition, glucosinolates and nitrogenous substances using FT NIR spectrometer Antaris II (Thermo Scientific, Madison, WI, USA) equipped with interferometer and the integrating sphere working with the technique diffuse reflectance. Samples weighing about 0.8 - 1 g were placed into a measuring cuvette. Spectra were obtained in the range of 12,500 - 4000 cm^{-1} at resolution 2 cm^{-1} and the number of scans at 128. Calibration models NIRS for predicting oil content, oleic acid, linoleic, linolenic, erucic acids, glucosinolates, nitrogen compounds were generated from samples collected as a part of the breeding process and the collection of the Czech National Program for conservation and utilization of plant genetic resources and biodiversity (NP), which were simultaneously analyzed by laboratory reference methods (gas and liquid chromatography, extraction) and were collected over a period of four years.

5.9 Microspore Culture

Donor plants were grown in the soil in a greenhouse for 4 weeks. Then, the plants with 3 to 4 developed leaves were transferred to a vernalisation chamber at 4°C, and 12h photoperiod (irradiance 20.4 μmol/m2/s) for subsequent 7-8 weeks. Finally, the vernalized plants were grown in the greenhouse up to the stage of flower bud formation and served as donors for flower bud collection and microspore isolation.

Liquid NLN-13 medium (Lichter 1982) with 13% sucrose was used for liquid washing medium, microspore culture, subsequent embryo induction and initial development. Media for embryo development (MD) and for plantlet regeneration (MR) were used as modified B5 media (Gamborg *et al.* 1968). MD medium contained increased KNO_3 up to 3000 mg/l and $CaCl_2.6H_2O$ up to 1100 mg/l with addition of amino acids (800 mg/l glutamine, 100 mg/l serine), 20 g/l of sucrose and growth regulators: 0.2 mg/l BAP and 0.2 mg/l IAA. In MR medium, the composition of macroelements will be modified as

follows: 1650 mg/l NH_4NO_3, 1900 mg/l KNO_3, 170 mg/l $KH_2PO_4.2H_2O$, 370 mg/l $MgSO_4$.7H2O; the medium supplemented with 2 mg/l of glycine and 10 g/l of sucrose. MS rooting solid medium (Murashige & Skoog 1962) with addition of 0.03 mg/l NAA contained 10 g/l of sucrose. pH of all media was adjusted with KOH to 5.8–6.0. NLN-13 medium was filter-sterilized (0.22 μm filter, Millipore) under sterile conditions in air laminar flow. Liquid NLN-13 (washing) and solid MS, MD and MR media were sterilized by autoclaving (121°C, 15 min).

The procedure of microspore isolation was done according to Klíma *et al.* (2004), Smýkalová *et al.* (2006) and Vyvadilová *et al.* (2008). Unopened flower buds 2.5–3.5 mm in length at the mid-uninucleate stage of pollen development were surface sterilised with 70% ethanol for 2 min, first 10 min and then washed three times with cold sterile deionized water. The flower buds were carefully crushed using a glass stick in a small volume of the washing NLN medium. The crushed flower buds were filtered through 44 μm and 70 μm nylon screen meshes, diluted with washing medium to the final volume of about 10 ml and centrifuged (1000 g) for 10 min. The supernatants were decanted and the pellets were washed twice with washing medium by centrifugation for 5 min. The obtained pellets containing microspores were diluted with NLN-13 medium (Lichter 1982) up to a density of about 75 000 to 100 000 microspores per 1 ml of media. The microspore cultures were sampled each 5 ml per 60 mm Petri dish.

The isolated microspores were incubated in NLN-13medium at 30°C, in darkness for 10 to 14 days. After visual detection of small embryoids about 1–1.5 mm in size, the Petri dishes were transferred onto a gyratory shaker (60 rpm) and cultured on the same medium at 26 ± 1°C, 16 h photoperiod, irradiance 20.4 μmol/m2/s for further 10 to 14 days. The first signs of root elongation and root hair formation were recorded at the end of this culture period.

The fully morphologically developed embryos about 5 mm in length were transferred onto MD medium in a culture room at 20–22°C, 16 h photoperiod (cool-white fluorescent tubes, irradiance of 20.4 μmol/m2/s) for 7 to 10 days. After removing cotyledonary leaves (Klíma *et al.* 2004), embryos were transferred onto MR medium at the same culture conditions as mentioned above for further 3 to 4 weeks. The germinating embryos were transferred onto the rooting medium MS for 4 to 6 weeks to support root growth. Then the regenerated plants were transferred to the soil and grown in a greenhouse

up to the stage of at least 4 leaves, and then subjected to vernalisation in a vernalisation room (4°C, 12 h photoperiod, irradiance 56 μmol/m2/s) for 7-8 weeks. Vernalized plants were grown up to flowering and seed setting in a greenhouse.

5.10 Assessment of Other Agronomical Characteristics

Field and/or glasshouse experiments were realized at VÚRV, v.v.i. Pollen fertility, morphological and physiological traits (phenotype, spring/winter type) and other agronomic characteristics were evaluated and expressed as a mean value from at least five plants.

5.11 Statistical Analyses

To prepare the sets of measured values for the analysis of variance and calculation of correlations, the percentage data will be converted via angular transformation to follow normal distribution of errors (Gómez & Gómez 1984). Analytic software STATISTICA (StatSoft, Inc., Tulsa, OK, USA) will be used for computing ANOVA procedures, Tukey HSD intervals (or Fisher's LSD) and Pearson product moment correlations between each pair of variables.

6 RESULTS

6.1 Optimization of Embryo Rescue on Conventional Oilseed *Brassica napus* L.

The siliquae growth and development of oilseed *Brassica napus* L. was evaluated in four modern cultivars, Benefit, Cadeli, Californium, Navajo and Viking. In total, 243 embryos were obtained from culture in liquid NLN-13 medium, MS and MD in the same culturing conditions of microspore cultivation under light (at first about two weeks in a gyratory shaker (60 rpm) and on the same medium at 26 ± 1°C, 16 h photoperiod, irradiance of 20.4 μmol/m2/s for about 10-14 days. Embryos taken from 28 to 16 days old siliquae after pollination were developed normally into a plant if cultivated on any medium, except for MS at 16 days in the variant with the Californium genotype, which had no yield at all. Embryos harvested at 28 DAP did not presented problem to germinate *in vitro*. Performance of embryo culture reduced to an average of 2 per siliqua at 21 days and 0.5 at 16 days.

Cultivation of 14 days old or older ovules, from all chosen cultivars, induced embryo development in liquid media. No embryos were produced with this method from ovules harvested earlier than 14 days. In general, cultures with normal solid differentiation medium (MD) and with solid regeneration medium (MR) were recorded the most suitable and the second best suitable for embryo production rate, respectively, at all ages of harvested siliqua. It was difficult to evaluate ovules performance at 10 DAP or earlier; however, they produce embryos only in differentiation medium. At 7 DAP ovules performed only few embryos in Navajo and Californium. Subculture of tested embryos was done according to Klíma *et al.* (2004). No embryo could be cultured from ovule culture of siliquae harvested 4 DAP.

6.2 Resynthesis of *Brassica napus* L.

Most of genotypes, used for interspecific hybridisation, already 14 days to 10 DAP presented siliques with wrinkled and yellowish ovules. Therefore, 11 DAP was set as the latest time on average for the interspecific crosses of these materials as proper time to harvest. Crossability and embryogenic potential were evaluated with the optimized protocol by means of ovule culture in different one-sided crosses with *B. rapa* as the

female component, 11 DAP. In the following experiments, the optimized protocol was tested, and the produced hybrids were tested also by morphology, ploidy, viability, seed set and seed quality.

6.2.1 Experiment One: On Embryo Rescue Techniques

Hilgert-Delgado A, Klíma M, Viehmannová I, Urban MO, Fernández-Cusimamani E, Vyvadilová M (2015). An Effective Method of Resynthesized Oilseed Rape Embryo Production Via Ovule Culture Of Different Crosses Between Winter Turnip Rape And Curly Kale. Plant Cell Tiss Orga 120:191-201

Declaration of Authorship: I, as the first author, got involved or realized myself, at least partially, every single aspect of the paper.

It was possible to identify the most productive crosses of the latter experiment where the regeneration was achieved in 23 out of 24 combinations. The pollination efficiency (i.e. the setting of siliquae) and the number of embryos per siliqua were studied. Results of this method in the majority of crosses were better than in similar, already published experiments and provided a simplified and less laborious method. Due to highly significant differences in relative DNA content between all hybrid combinations and their respective parental components it was possible to reliably assess the hybrid nature of all regenerants via flow cytometry. As the occurrence of the self-pollinated and/or somatic-tissue regenerated female parent was not detected, the hybridity of all regenerants was reliably verified. Morphological assessment of regenerated plants showed typical characteristics originating from both parental components, and further corroborated the results of flow cytometric analysis.

6.2.1.2 Crossing and *in vitro* Culture

A total of 1,670 siliquae were produced after the pollination of 2,304 buds. Although we found a relatively large spread between the worst and best mean pollination efficiency (50.9% - 91.7%, Fig. 5*a*), no significant effect of a cross combination and no differences between combinations were detected, according to non-parametric Kruskal-Wallis one-way analysis of variance and multiple mean comparisons at $P \leq 0.05$. The average loss of siliquae (due to handling and/or contamination) was 16.3%. The first embryos appeared after approx. two weeks of culture, depending on the cross combination and/or replication; twenty-three out of twenty-four combinations successfully produced

embryos when counted on the 35th day of culture (Fig. 5*b*). In total, 673 cotyledonary embryos and six calli from 1,332 siliques were produced without contamination (i.e. 0.51 embryos/siliquae, and 0.34 embryos/bud, respectively).

Specifically, the crosses SV×KA, AR×FR, SV×KZ, and IZ×SC (with means of 1.73/1.57, 1.16/0.98, 0.78/0.67, 0.92/0.52 HSR/HPR, respectively; Fig. 5*b*) had the highest performance; while BR×KA, GW×FR, and BR×KZ (with means of 0.10/0.07, 0.17/0.07, 0.13/0.11 HSR/HPR, respectively) exhibited the lowest average yield. The GW×KA cross produced no embryos. The Kruskal-Wallis test did not confirm any significant effect of a cross combination on embryo production, nor differences between combinations per siliquae, or per bud (except for GW×KA, the non-productive combination; and SV×KA, the most productive). Nevertheless, we were able to identify several combinations, which were among those with the highest yields in each replication. The highest absolute value in one replication was achieved in the cross SV×KA with 2.66 HPR and 2.81 HSR (Fig. 5*b*).

Weak, but significant, positive correlations (r = 0.26* and r = 0.50*) were detected between the pollination efficiency with HSR as well as HPR at $P < 0.05$ (Fig. 5*c*). Regression analyses identified the relationship between the aforementioned variables as substantially linear (Fig. 5*c*). In general, the higher the pollination efficiency (i.e. the setting of siliquae), the higher the average yields of embryos per individual siliqua or bud, and *vice versa*.

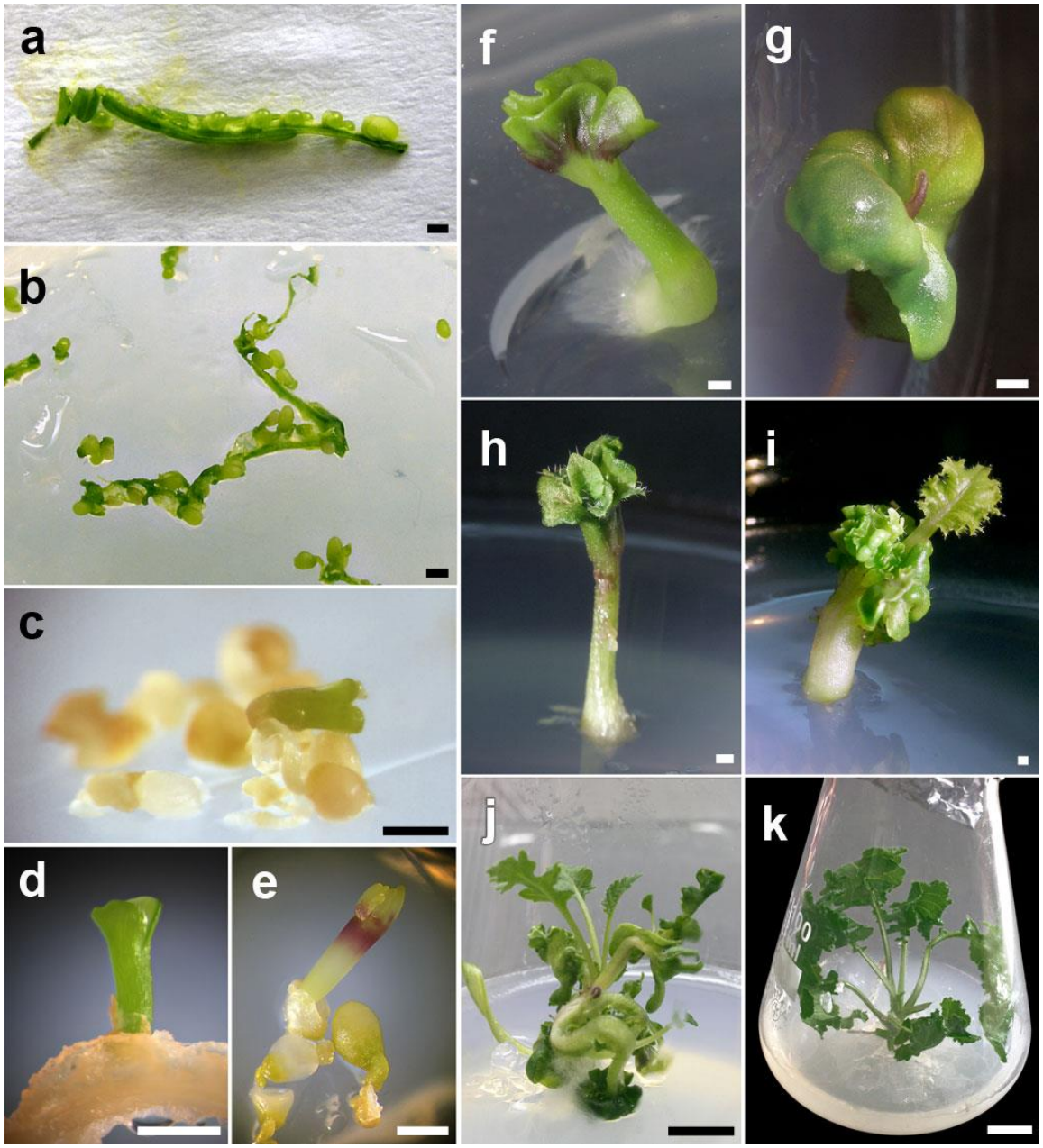


Fig. 4 *In vitro* plant regeneration from excised immature ovules

Halved siliqua with ovules eleven DAP (*a*). Ovules (individual or attached to the septum), placed on differentiation medium (*b*). Rescued embryos from crosses between *B. rapa* (female parent) and *B. oleracea* (male p.); – Arktus × Kaderavek Zelený after 26 days of culture (regeneration from an individual ovule (*c*). – Svalöfs Duro × Kaderavek Zelený after 33 days of culture (regeneration from an ovule attached to the septum) (*d*). – Izumrudnaja K193 × Scarlet after 42 days of culture (regeneration from an individual ovule (*e*). – Bulharska × Scarlet cotyledonary embryo after 50 days of culture (on fresh differentiation medium) (*f*). Regeneration of first true leaf from embryos on regeneration

medium; – the Brachina × Scarlet cross after 55 days of culture (*g*). – Arktus × Kapral cross after two months of culture (*h*). – Bulharska × Frosty cross after two months of culture (*i*). Regeneration of whole plants; – Svalöfs Duro × Frosty plant after three months of culture on regeneration medium (*j*). – Grubers Winterrübsen × Scarlet plant after four months of culture on MS medium (*k*). For *a–i*, bar = 1mm; for *j*, bar = 5 mm; for *k*, bar = 10mm

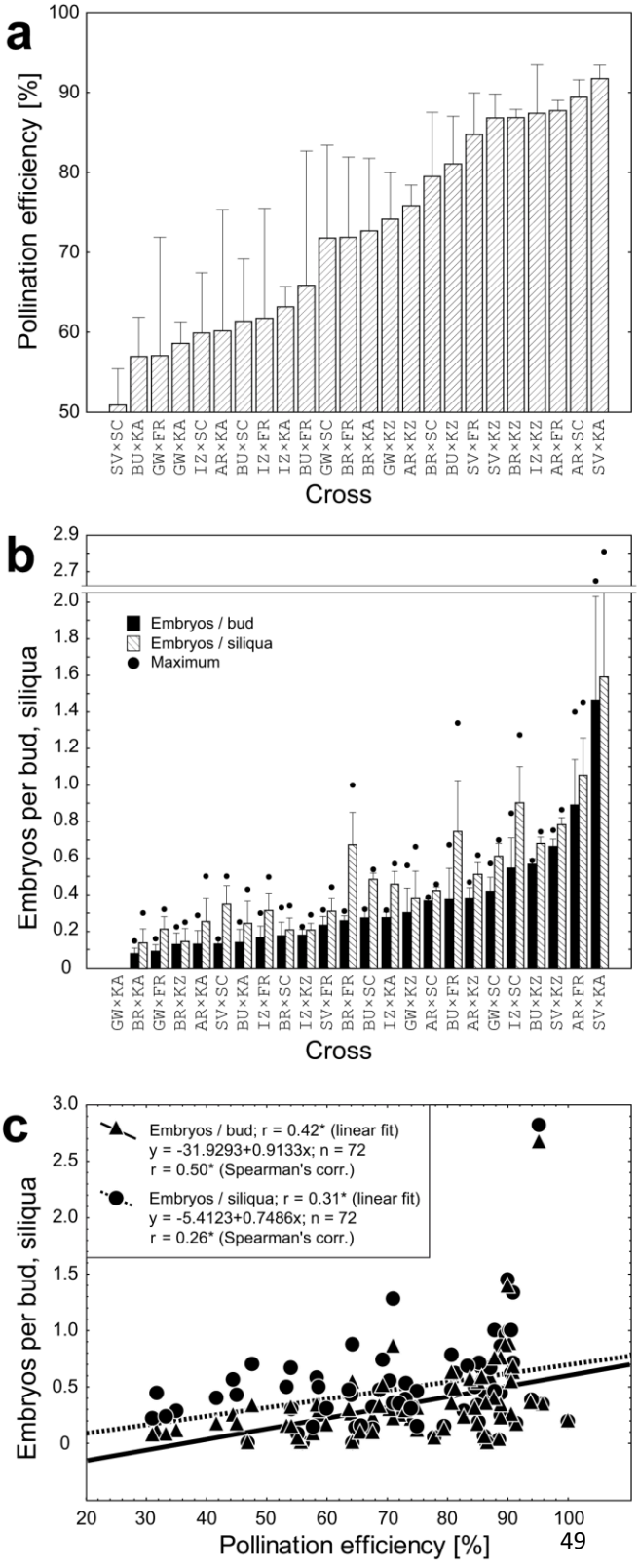


Fig. 5 Results of interspecific crosses. **a** Pollination efficiency, **b** HPR and HSR of RS embryos, **c** relationships between pollination efficiency and HSR or HPR, from selected crosses between *B. rapa* (female parent) and *B. oleracea* (male parent).

Pollination efficiency = the ratio between the number of siliquae with at least one ovule developed and the number of flower buds pollinated (**a**). *The columns denote means ± standard errors; for genotypes abbreviations see Table 2. Correlation coefficient significant at $P < 0.05$ (**c**).

6.2.1.3 Flow Cytometry, Leaf Morphology, and Chromosome Counts

Flow cytometric analyses revealed substantial interparental differences in their relative DNA content (Fig. 6 *a*,*b*). According to the statistical analysis, these differences were highly significant for all genotypes tested (Table 5). Thus, we were able to distinguish both parental components from respective hybrids and *vice versa* in histograms (Fig 6*c*). Moreover, G2 and even G1 peaks were still clearly distinguishable; this also being the case in the female component and in the hybrid of the combination with the smallest difference in relative DNA contents (Fig. 6*d*). This fact enabled us, using bulk samples from up to five regenerants, to identify the potential occurrence of a self-pollinated (or from somatic tissue) regenerated female component. In addition, this assumption has been experimentally confirmed (Fig. 6*e*). Nevertheless, since analysis of 526 regenerants did not reveal any such female component, all regenerants were assumed to be of hybrid origin (Fig. 6*f*). Results from flow cytometric analyses were further assessed via morphological comparisons of female, hybrid, and male true leaves. Hybrids possessed signs of both parents (Fig. 7*d-g*). Typical characteristics, originating only from the male parent (i.e. curliness, eventually traces of purple colour), clearly demonstrate the hybrid origin of the regenerants. The evaluations based on FCM analyses and morphological assessment corresponded well with the cytological observations (Fig. 7*a-c*).

Table 5 DNA ratios of ten parental components. Isolated nuclei were stained with DAPI, and *Zea mays*, cv. CE-777 (2C = 5.43 pg) was used as an internal standard

Accession (Cultivar)*	Genome composition	DNA ratio (mean ± SD)
B. rapa		
AR	AA	0.301 ± 0.002 a
BR	AA	0.305 ± 0.002 a
BU	AA	0.301 ± 0.002 a
GW	AA	0.304 ± 0.002 a
IZ	AA	0.300 ± 0.003 a
SV	AA	0.298 ± 0.001 a
B. oleracea		
FR	CC	0.410 ± 0.003 b
KZ	CC	0.409 ± 0.003 b
KA	CC	0.404 ± 0.003 b
SC	CC	0.339 ± 0.001 b

Mean values in a column, followed by different letters, are significantly different according to the Tukey's HSD test ($P < 0.05$). *For unabbreviated cultivar names, see Table 2.

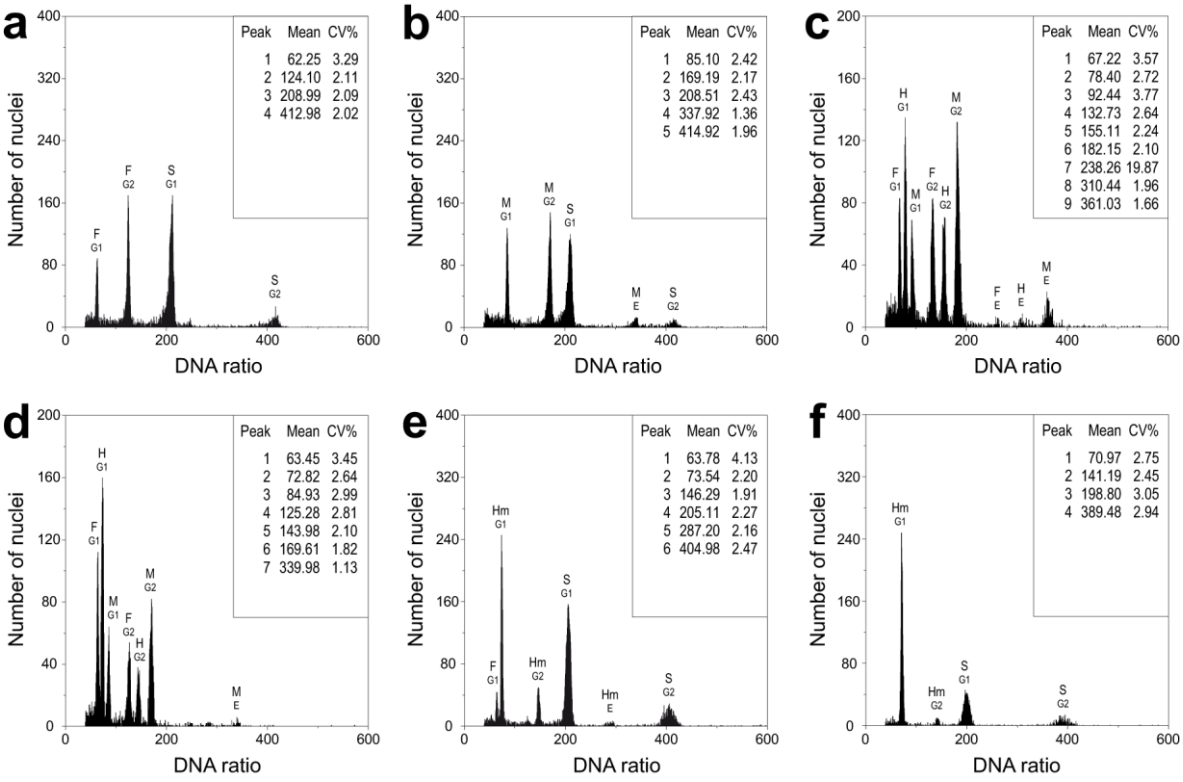


Fig. 6 Assessment of hybrid origin via flow cytometry. Representative flow cytometric histograms of parental components; **a** *B. rapa* ssp. *oleifera* f. *biennis* (female component) analysed together with the internal standard (*Zea mays*). **b** *B. oleracea* convar. *acephalla* var. *sabellica* (male component) analysed together with the internal standard (*Zea mays*). **c** Simultaneous analysis of parental components with their hybrid. **d** Simultaneous analysis of parental components with their hybrid. **e** Artificially prepared analysis of four regenerated hybrids analysed together with female component (*B. rapa* ssp. *oleifera* f. *biennis*) and the internal standard (*Zea mays*). **f** Simultaneous analysis of five regenerated hybrids analysed together with the internal standard (*Zea mays*). F, female component; M, male component; S, internal standard; Hm, hybrid mixture; G1, nuclei in G1 phase; G2, nuclei in G2 phase; E, peaks representing endopolyploid nuclei

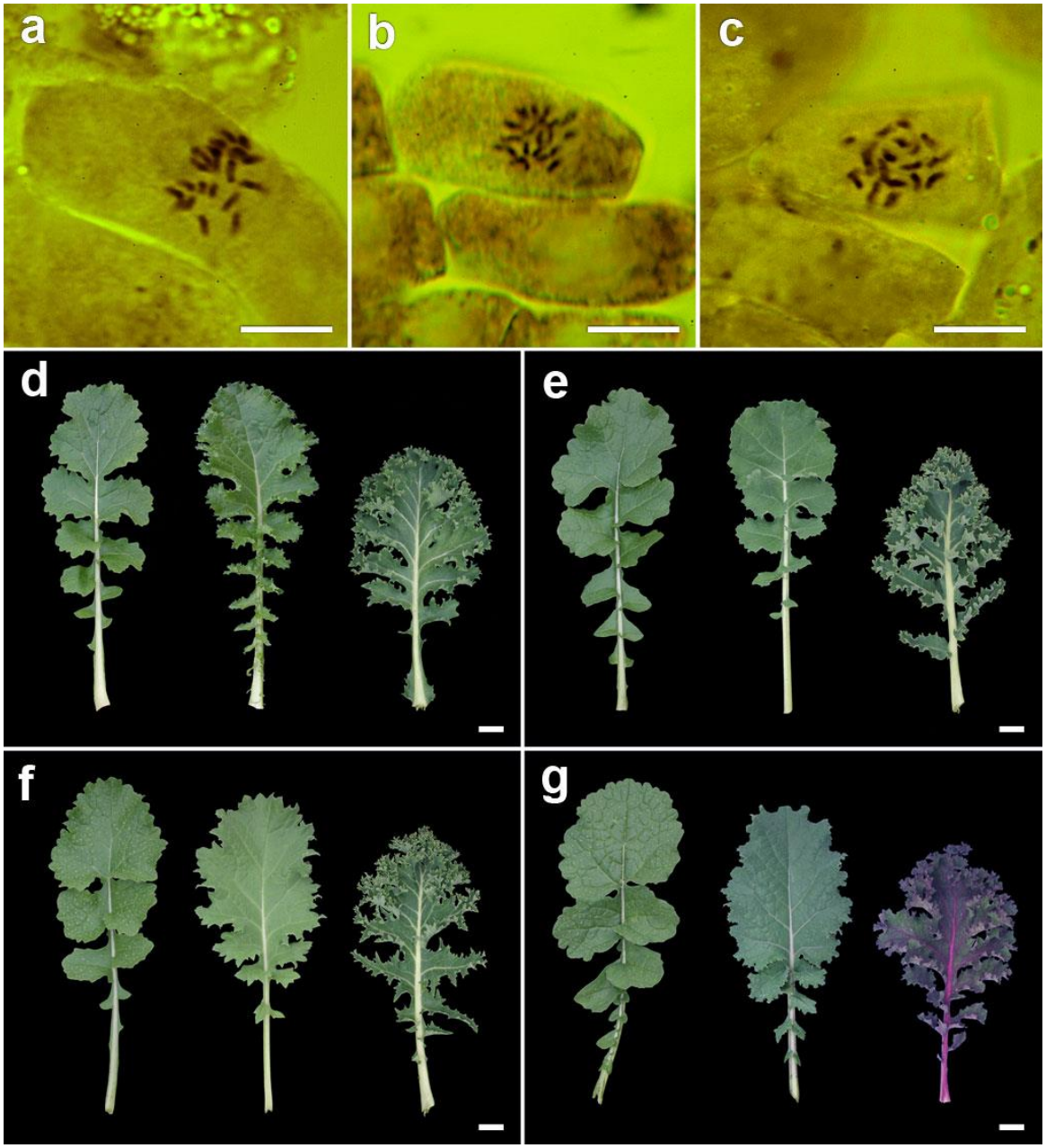


Fig. 7 Cytological and morphological assessment of hybrid origin. Lacto-propionic-orceine stained chromosomes of female (2*n* = 20, **a**) and male (2*n* = 18, **c)** components, and their hybrid (*n* = 19, **b**). Morphological comparison of true leaves of female (left) and male (right) components, and their hybrid; **d** Brachina × Kaderavek Zelený; **e** Svalöfs Duro × Kapral; **f** Izumrudnaja K193 × Frosty ; **g** Bulharska × Scarlet. [For **a-c**, bar = 10μm; for **d-g**, bar = 10 mm]

6.2.2 Experiment two: On Crossability of Wider Germplasm

Hilgert-Delgado A, Klíma M, Viehmannová I, Fernández Cusimamani E. Effective Techniques for Resynthesized Rapeseed Production of Contrasting Components via Ovule Culture and Flow Cytometry (2014). Tropentag, 17-19.09, 2014. Czech University of Life Sciences Prague Czech Republic

Declaration of Authorship: I, as the first author, got involved or realized myself, at least partially, every single aspect of the paper.

Fig. 8 The cross of turnip rape V17 and cabbage Vysocké AIK

A **B** **C**

A Flowering stage - Hybrid with intermediate phenotype in the middle. **B** Detail of the bulb heritated from the turnip rape. **C** Leaf stage - Hybrid on the left.

6.2.2.1 Crossing and *in vitro* Culture

In this experiment, the resynthesis crosses were tried on turnip rape breeding material V17 (*Brassica rapa* var. *rapa*) with pollinised with Cabbage accession Vysocký AIK line; spring oilseed turnips (*B. rapa* var. *oleifera*), Ante-12, Ante-27, Jumbuck and Evvisa with cabbage assessions Zakamenné and Vysocký AIK line (except on Evvisa). Parallely with the second experiment, accessions of Arktus, Brachina, Bulharska, Grubers Winterrübsen, Izumrudnaja K 193, Svalöfs Duro were crossed with Pentland Brig and Nero di Toscana, while Brachina and Rapido with Vates, Slezsky Krajový with Nero di Toscana and Brachina, Izumrudnaja K 193, Ludowy and Svalöfs Duro with Zakamenné. This results together with the second experiment (shown bellow separately) had an average production rate of 0.41 embryos/siliquae and 0.32 embryos/bud, respectively. The first embryos appeared after approx. two weeks of culture, depending on the cross combination and/or replication; forty-two out of fifty combinations successfully produced embryos

when counted on the 35th day of culture. Therefore, successful regeneration of embryos was achieved in overall 84% of crosses.

Table 6 Crossability of individual combinations. Assessed by means of pollination efficiency (P. E.), hybrid siliquae ratio (HSR) and hybrid production rate (HPR)

♀	♂	P. E.	HSR	HPR
Arktus	Frosty	90,00	1,45	1,40
Brachina	Frosty	90,63	1,00	0,25
Bulharska	Frosty	90,91	1,33	0,67
Grubers Winterrübsen	Frosty	85,00	0,33	0,17
Izumrudnaja k 193	Frosty	84,62	0,50	0,30
Svalöfs Duro	Frosty	91,43	0,18	0,04
Arktus	Kapral	88,57	0,03	0,10
Brachina	Kapral	86,11	0,06	0,06
Bulharska	Kapral	46,88	0,30	0,16
Grubers Winterrübsen	Kapral	64,29	0,00	0,00
Izumrudnaja k 193	Kapral	58,33	0,58	0,32
Svalöfs Duro	Kapral	95,24	1,81	1,65
Arktus	Kadeřávek zelený	81,25	0,62	0,48
Brachina	Kadeřávek zelený	86,49	0,18	0,22
Bulharska	Kadeřávek zelený	90,63	0,62	0,54
Grubers Winterrübsen	Kadeřávek zelený	64,71	0,14	0,09
Izumrudnaja k 193	Kadeřávek zelený	100,00	0,19	0,19
Svalöfs Duro	Kadeřávek zelený	88,89	0,85	0,75
Arktus	Scarlet	93,94	0,38	0,36
Brachina	Scarlet	67,65	0,13	0,09
Bulharska	Scarlet	70,37	0,56	0,29
Grubers Winterrübsen	Scarlet	87,10	0,67	0,58
Izumrudnaja k 193	Scarlet	70,97	1,27	0,85
Svalöfs Duro	Scarlet	57,69	0,14	0,15
Arktus	Nero di Toscana	55,56	0,00	0,00

♀	♂	P. E.	HSR	HPR
Brachina	Nero di Toscana	84,11	0,41	0,15
Bulharska	Nero di Toscana	48,14	0,00	0,00
Grubers Winterrübsen	Nero di Toscana	65,87	0,39	0,35
Izumrudnaja k 193	Nero di Toscana	86,48	0,00	0,00
Svalöfs Duro	Nero di Toscana	64,25	0,00	0,00
Arktus	Pentland Brig	67,67	0,15	0,15
Brachina	Pentland Brig	75,56	0,32	0,19
Bulharska	Pentland Brig	72,23	0,18	0,14
Grubers Winterrübsen	Pentland Brig	56,00	0,00	0,00
Izumrudnaja k 193	Pentland Brig	55,56	0,00	0,00
Svalöfs Duro	Pentland Brig	46,85	0,00	0,00
Slezsky krajový	Nero Di Toscana	67,51	0,12	0,03
Brachina	Vates	86,11	0,06	0,06
Rapido	Vates	67,65	0,13	0,09
V17	Vysocké AIK	84,62	0,14	0,15
Jumbuck	Vysocké AIK	84,57	0,52	0,48
Ante-12	Vysocké AIK	91,25	1,15	1,28
Evissa	Zakamenné	89,87	0,37	0,52
Ante-12	Zakamenné	89,66	0,74	0,62
Ante-27	Zakamenné	73,17	0,66	0,38
Jumbuck	Zakamenné	88,89	0,14	0,32
Izumrudnaja k 193	Zakamenné	69,23	0,71	0,12
Svalöfs Duro	Zakamenné	85,71	0,42	0,33
Ludowy	Zakamenné	79,41	0,42	0,15
Brachina	Zakamenné	90,91	0,34	0,53

6.2.3 Experiment Three: On Viability to Fertile Plants

Hilgert A, Klíma M, Urban M (2014) Effective resynthesis of rapeseed via ovule culture technique. Úroda 12/2014, vědecká příloha časopisu (in Czech language).

Declaration of Authorship: I, as the first author, got involved or realized myself, at least partially, every single aspect of the paper.

6.2.3.2 Crossing and *in vitro* Culture

A total of 502 siliquae were obtained by crossing the 600 buds.Resynthesized embryos were obtained in each of the ten crossed combinations. In total, there were obtained 256 embryos (up to the 35th day since the beginning of the ovule culture). Pollinisation of the siliquae with at least one embryo happened in 84% of the buds. An average of 0.49

embryos per silique were obtained, respectively 0.43 embryos per bud (for more results see Table 7).

Table 7 Success of each crossed combination: by expressed crossability (% of siliquae by pollinated buds), the average number of embryos per silique; Hybrid siliquae ratio (A) and per pollinated bud; Hybrid production rate (B)

Type	♀	♂	**Crossability [%]**	**HSR A**	**HPR B**
S	Ante	Vysocké AIK	91	1.15	1.28
W	Brachina	Zakamenné	91	0.34	0.53
S	Evissa	Zakamenné	90	0.37	0.52
S	Jumbuck	Vysocké AIK	85	0.52	0.48
S	Ante	Zakamenné	73	0.66	0.38
W	Svalöfs Duro	Zakamenné	86	0.42	0.33
S	Jumbuck	Zakamenné	89	0.14	0.32
S	V17	Vysocké AIK	85	0.14	0.15
W	Ludowy	Zakamenné	79	0.42	0.15
W	Izumrudnaja k 193	Zakamenné	70	0.71	0.12

S - Spring, W - Winter crop; male components (Vysocký AIK and Zakamenné) are winter crops.

Average crossability ranged from 70% to 91%. The highest average Hybrid production ratio (1.28) was achieved in the combination of Ante × Vysocký AIK; in this combination were also produced 77 hybrids, the highest number of embryos.

Fig. 9 Turnip rape, spring rapeseed and resynthesis with cabbage.

A  **B**

Comparative morphology of resynthesized rapes (middle) and its parents (female component is always on the left). **A** - V17 × Vysocký AIK; **B** - Ante × Vysocký AIK.

6.2.3.3 Colchicine Treatment Results

On average, 51% of colchicine treated regenerants formed flower stalks with fertile flowers; after self-pollination of flowers or flower buds, respectively crossbreeding of bud with fertile rape pollen, only 38% of these plants set seeds.

6.2.3.4 Colchicine Treatment Extention to Previous Experiments

Colchicine diploidization treatment was realized for the rest of materials, which were not mentioned in experiment three, but achieved in and after experiments one and two. In total, forty two combinations and over 800 plants lines have been colchicinated. From one to ten lines per combination were kept *in vitro* till self-pollinated seeds were obtained. Clones from the resynthesized crossed combinations for diploization were multiplied by 20 to 30 plants, from which 10 to 20 rooted plantlets were taken out of the flasks for *in vivo* polyploidization protocol with colchicines solution as described in chapter 5.4. Results of polyploidization are characterized by fertile / unfertile flower properties and siliquae/seed set with self pollination in isolated flower or bud or pollination by other rapeseed pollen donor.

On current basis, I state that the average colchicination rate and seed sed in all 42 mentioned cross combinations have given, up to date, similar values as the above shown results. Self-pollination crosses as well as with elite cultivars are continuously made in order to gather enough material for further analyses.

Fig. 10 Resynthesized *Brassica napus* clones after colchicine treatment in acclimatization to *in vivo* conditions

6.2.4 Experiment Four: On Seed Quality (Preliminary Results)

Adapted from the Jrec article: Quality parameters in seeds of selected resynthesized rape and quality donors' determined using Near-infrared Spectroscopy (NIRS). Hilgert-Delgado A, V Vrbovský, Endlová L Klima M. 14th Conference of Plant Experimental Plant Biology 2015. Proceedings of abstracts of poster seccion (in Czech) P10-1: 92.

Declaration of Authorship: I, as the first author, collaborated in every single aspect of the paper except for the NIRS analyses, which were done in collaboration with at OSEVA Development and Research Ltd.

6.2.4.2 Analyses of Seed Quality Parameters

The seed quality parameters of resynthezised genotypes, their parental components, the quality donors and the cross of RS genotypes with a quality donor were compared using the method of NIRS (near infrared spectroscopy). Seed of RS genotypes pollinized by bees were also analysed for view of an approximated quality of the crosses between random RS cultivars. In both of the selfed RS genotypes were found high content of antinutriotional compounds i.e. glucosinolates (69.0 to 78.9 micromol per g dry weight at 8% moisture), erucic acid (19.4 to 40.9%) and low oil content (32, 0 - 35.0%) compared to donors of quality (~ 14 micromols, ~ 0%, respectively ~ 41%). For all the values see table 8.

Table 8 Seed quality parameters of two resynthesized lines with related cultivars

RS Cross x Donor		Humidity (%)	Oil at 8% humidity (%)	GSL at 8% humidity (µmol/g)	Oleic acid (%)	Linoleic acid (%)	Linolenic acid (%)	Erucic acid (%)	Nitrogen compounds (%)
Jumvys	Ladoga	6,45	34,34	64,62	32,01	13,32	8,54	31,81	27,16
Jumvys	-	6,45	35,03	69,00	24,87	13,81	8,21	40,92	26,17
Jumbuck	-	6,06	31,89	-	72,12	27,12	10,45	stopy	-
Jumvys	By bees	6,43	33,14	73,20	26,37	12,17	7,93	35,51	26,71
RS Cross x Donor		**Humidity (%)**	**Oil at 8% humidity (%)**	**GSL at 8% humidity (µmol/g)**	**Oleic acid (%)**	**Linoleic acid(%)**	**Linolenic acid (%)**	**Erucic acid (%)**	**Nitrogen compounds (%)**
Cadeli	-	6,49	40,97	14	66,24	17,72	7,81	stopy	22,07
Antvys	Cadeli	6,44	34,93	49,34	48,11	13,51	8,97	5,067	27,47
Antvys	-	6,17	32,01	78,9	35,63	13,39	8,88	19,371	28,01
Ante	-	5,66	34,73	-	70,28	23,89	10,84	stopy	-
Antvys	By bees	6,125	33,285	72,71	37,885	12,935	8,385	21,45	27,92

6.2.5 Experiment Five: On Selected Seed Quality Parameters

Adapted from the Jrec article: Hilgert-Delgado A, Vrbovský V, Endlová L, Urban MO, Klíma M (2015) Development of New Genotypes and Evaluation of Selected Seed Quality Parameters in Resynthesized Rape. Úroda 12/2015, vědecká příloha časopisu (in Czech language).

Declaration of Authorship: I, as the first author, collaborated in every single aspect of the paper except for the GC analyses, which were done in collaboration with at OSEVA Development and Research Ltd.

Table 9 Representation of fatty acids in the oil of the starting materials. RS genotypes and their F_1 hybrids with conventional varieties Ladoga

Fatty acids [%]	**Palmitic** 16:0	**Stearic** 18:0	**Oleic** 18:1	**Linoleic** 18:2	**α-Linolenic** 18:3	**Arachidic** 20:0	**Eicosenoic** 20:1	**Eicosedienoic** 20:2	**Behenic** 22:0	**Erucic** 22:1	**Docosadienoic** 22:2	**Docosahexaenoic** 22:6	**Lignoceric** 24:0
Parental genotypes													
Ante (ANT)	3.4	1.4	59.0	22.1	12.4	0.3	1.0	0.1	0.1	traces	traces	0.1	0.2
Jumbuck (JUM)	3.8	1.6	53.2	25.7	13.7	0.4	1,0	0.1	0.2	0.2	traces	0.1	0.3
Brachina (BRA)	2.9	1.0	25.8	18.5	9.2	0.6	9.0	0.6	0.7	30.2	0.4	0.2	1.2
Izumrudnaja (IZU)	3.0	0.9	15.4	17.7	9.5	0.6	8.8	0.7	0.7	40.9	0.7	0.2	1.4
V17 (VOD)	2.7	1.1	14.5	14.8	8.7	0.8	10.9	0.7	0.8	43.5	0.6	0.3	1.2
Vysocké (VYS)	3.8	0.6	27.9	10.4	8.2	0.4	9.2	0.3	0.4	37.4	0.3	0.1	1.3
Vates (TES)	3.8	0.6	18.8	15.0	9.4	0.4	8.3	0.5	0.5	41.3	0.5	0.2	1.2
Kapral (PRAL)	5.2	0.6	15.9	20.0	13.1	0.3	6.1	0.7	0.4	35.5	0.8	0.2	1.3
Scarlet (SCA)	6.0	0.7	10.2	16.2	12,7	0.5	6.8	0.7	0.5	43.1	1.2	0.3	1.3
Ladoga (L)	3.9	1.5	64.8	19.4	8.4	0.2	0.9	0.2	0.1	0.3	0.1	0.1	0.1
RS genotypes													
IZUSCA	5.5	1.0	32.0	16.0	14.2	0.4	12.6	0.4	0.1	17.3	0.1	traces	0.2
JUMVYS	3.5	1.0	29.4	13.1	8.4	0.5	14.3	0.5	0.2	27.5	0.2	0.1	1.4
ANTVYS	3.0	1.2	29.3	14.7	12.2	0.5	12.3	0.5	0.2	24.6	0.1	0.1	1.2
BRAPRAL	2.4	1.2	25.1	10.0	6.2	0.8	14.0	0.3	0.4	37.9	0.2	0.2	1.1
VODVYS	2.5	0.7	23.6	12.7	11.0	0.4	10.6	0.5	0.3	36.0	0.3	0.2	1.2
BRATES	2.3	0.7	18.5	13.2	9.3	0.5	10.4	0.6	0.5	42.3	0.6	0.1	0.9
IZUKA	3.2	1.0	18.5	14.1	12.0	0.6	12.9	0.7	0.3	35.1	0.4	0.2	1.2
RS genotypes × Ladoga													
ANTVYS × L	3.6	1.8	40.7	15.6	8.5	0.6	14.8	0.3	0.2	12.9	traces	0.1	0.7
IZUSCA × L	6.0	1.6	36.6	18.7	8.1	0.6	12.6	0.4	0.2	14.7	0.1	0.1	0.3
BRAPRAL × L	3.7	1.5	33.5	11.9	8.9	0.6	16.5	0.4	0.2	22.0	0.1	0.1	0.6
VODVYS × L	5.8	1.2	30.8	20.4	16.1	0.4	9.6	0.5	0.1	14.8	0.1	traces	0.2
JUMVYS × L	3.3	1.1	24.8	13.8	9.5	0.7	13.5	0.5	0.4	30.8	0.2	0.2	1.1
BRATES × L	2.9	0.9	23.2	13.6	9.5	0.5	16.1	0.6	0.2	31.2	0.2	0.1	0.8
IZUKA × L	3.7	1.2	22.5	17.0	8.9	0.6	13.3	0.6	0.3	30.1	0.3	0.1	1.4

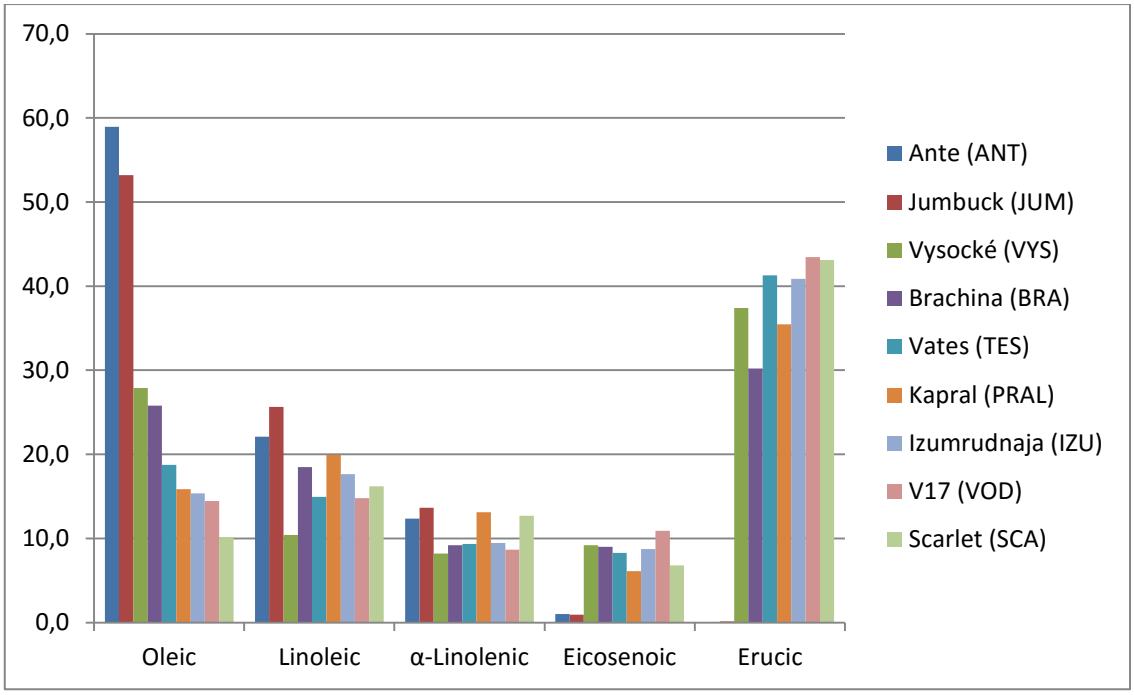
As in most of RS material only a limited number of seeds was available (due to RS genotypes autoincompatibility and therefore it was need to produce seed by bud

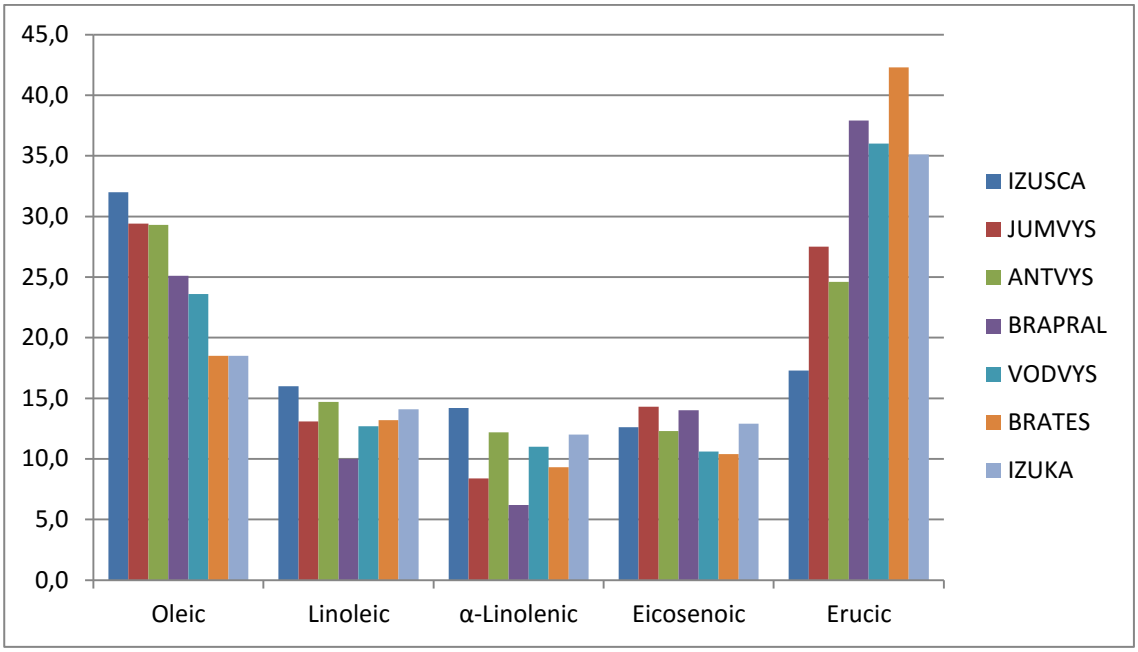
pollination), the analysis of fatty acids in all genotypes was carried out by gas chromatography, optimized for minimum weight of sample (Endlová *et al.* 2014).

6.2.5.2 Comparison of Seed Quality Parameters

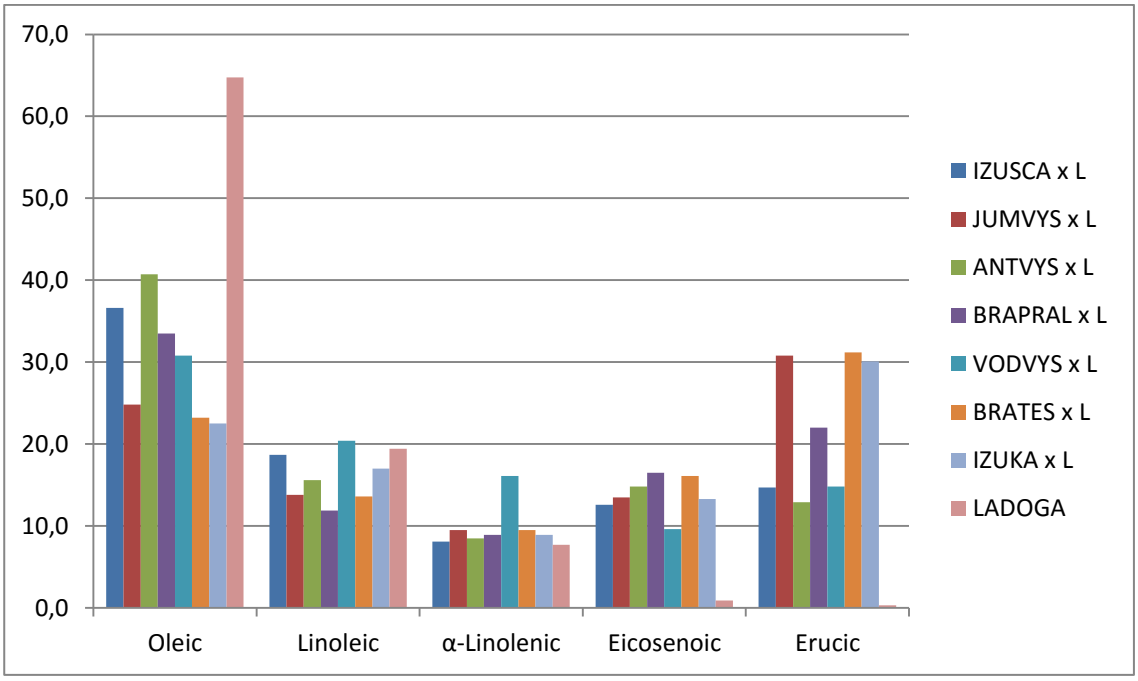
Analyses of fatty acid profiles showed that all RS materials were highly over the limit content of erucid acid content (17.3 – 42.3%, conventional varieties have up to 0.1%), below limit content of oleic acid (18.5 – 32.5%, 60 – 70%) and high content of eicosenoic acid (10.4 – 14.3%, normal varieties about 1%), see table 9. The two first mentioned acids concerted a mutual negative correlation (Fig. 11A). In the other fatty acids (linoleic, α-linolenic and eicosenoic acid) a significant correlation was not found in relation to the oleic and erucid acid (Fig. 11A). A positive correlation was found between linoleic and α-linolenic acids, Fatty acid profiles in RS materials of BRATES and IZUKA were close to high erucid acid oil (HEAR) rapeseed varieties, where erucid acid accounts for half of all the fatty acid content and oleic acid just about the 15%. Seeds of some of the RS rape materials showed a high percentage of α-linolenic acid in the oil content in comparison to the oil of conventional rapeseed (IZUSCA 14.2%, ANTVYS 12.2%, IZUKA 12.0%). The contents of the other fatty acids (linoleic acid, palmitic acid, stearic acid and 6 other minor acids) were comparable with the contents in the profiles of conventionally grown varieties.

Fig. 11 Representation of selected fatty acids (%) in oil. RS materials **A**, their crosses with conventional variety Ladoga **B** and parental components **C**





A



B

A C- Ranked in descending order according to oleic acid content; **B** - Ranked in order of genotypes in Fig. 11A

6.2.6 Experiment Six: On Embryogenic Capability in Microspore Culture and Seed Set of RS Material × Donor of Quality

This experiment was designed with my supervisor specialist Dr. Miroslav Klíma. The crosses and microspore culture stablishments were realized by myself and the embryo regeneration and post-culture was realized by the team technicians at the CRI in Ruzyně.

Out of the scope of this experiment, the obtained embryo were each numbered and their cotyledons were cut and frost for further studies aimed to find a possible correlation between their oil qualities and the seed qualitys of their regenerants. The resulting material will be also classified by ploidy, seet seed of fertile plants and their seeds will be analysed at OSEVA Development and Research Ltd. as in earlier experiments by NIRS and GS, in order to find the mentioned relationships from the respective analyses.

6.2.6.1 Results of Microspore culture

Six plants of each cross were cultivated according to the procedure for stablishment of microspore cultures with the use of Trifluraline. Best plants of each cross were selected to harvest best 25-30 buds in order to obtain microspore embryos in three replications and the obtained microspore embryos were passaged in different media and regenerated according to chapter 5.9. The embryogenic potential was analysed by optical measurements.

6.2.6.2 RS Crossability

These crossed genotypes were also materials were self - pollinised in flower and bud and labelled with conventional methods. Four of six crosses produced embryos, namely, Buzel × L with 108 embryos, Grusty × L with 135 embryos, Grusca × L with 64 embryos and Izuka × L with 108 embryos. These embryos were passaged to plantlets and taken to ex vitro conditions for further vernalisation and selfing in technical isolation. Most lines in all genotypes were viable.

At least one plant of each variant seed set normally or almost normally after selfing in flower in the combinations Buzel × L and Grusty × L. Only on plant of the cross Arkpet × L seed set few short siliquae, in bud and in flower, and sporadically, likely by genetic instability. Similarly was the case of Grusca × L plants, however, practically all the six plants were fertile, siliquea were obtained from selfing and also successful seed set with the cross of Ladoga was seen. The variants Izuka × L and Svazel × L were clearly autoincompatible and seed set after selfing only ocurred after pollinisation in bud (for details see table 10).

Table 10 Seed set quality and autoincompatiblity of RS material crossed with Ladoga

Cross	**Polinisation**		**Siliquae length**	**Autocompatibility**
Arkpet × L	Bud	*	Up to 4 cm	NO*
	Flower	*	Up to 4 cm	
	Control	*	Up to 4 cm	
Buzel × L	Bud	Very good	7 cm	YES
	Flower	Very good	7 cm	
	Control	Very good	7 cm	
Izuka × L	Bud	Good	6.5 cm	NO
	Flower	No	Up to 3.5 cm	
	Control	Very good	6.5 cm	
Grusty × L	Bud	Very good	6.5 cm	YES
	Flower	Good	Up to 4 cm	
	Control	Bad	6.5 cm	
Grusca × L	Bud	Bad	5 cm	NO
	Flower	No	No	
	Control	Bad	5 cm	
Svazel × L	Bud	Good	5.5 cm	NO
	Flower	No	No	
	Control	Very good	5.5 cm	

*Length of siliquae 3 weeks after polinisation. Further observations confirm that Arkpet × L plants can be autocompatible with sporadic seed set.

Fig. 12 Different values of siliquae length in the crosses of RS material with Ladoga.
A Complete siliquae with diferent lengths **B** Corresponding cut siliquae with seeds

*It can be noticed that the length and number of seeds does not always correlate.

7 DISCUSSION

Literature related to resynthesis of *B. napus* was first reviewed in order to approach a general idea of what have been done before and worked in oilseed rape resynthesis before the experiment was designed, and I came with the conclusion that in general it is always a poor embryo production what limits analyses. Therefore, general methods could be tested on the conventional winter rape as the material was easy to produce. It was tried to grow embryos, from microspore cultures in liquid cultures, similarly as Bennet *et al.* (2008) did to study the effects of silique age and the temperature growth conditions on the efficiency of hybrid production. However, due to further findings reviewed about specific genotype requirements and differences how they respond with different silique age and growth medium (Wen *et al.* 2008), it seemed that the most reliable results will be directly on the final material as a whole combination of factors. There were also embryos cultured from embryo rescue at different days. Once the embryos were visible, it was noticed favourable to take embryos out of the ovules or pre-culture to new fresh medium after a month according to klíma *et al.* (2004). Interestingly the number of seen embryos per siliquae, seen at 14 days after culture, was about half of the total number expected to be found (according to the average number of seeds per siliquae in these genotypes). This is in my opinion due to different stages of development within siliqua at the handling process, where only part of the embryos were above the limit of human eye recognition, growing at differant pase within one siliqua. Similarly, in microspore cultures, the embryos growth rate differs, and not all the embryos are passaged at the same time. The evaluated growth and development of the siliquae and correlation with the embryos inside served as a basis. It was also noticed that the morphological development was a little different from the embryos of microspore culture (see Fig. 2 and 4). Finally, it was assumed that each genotype had similar but not equal overall development and that growing conditions had a different effect on it. This situation helped to design the experiment for resynthesis (see Chapter 6.2).

Nevertheless, while in the experimets with conventional rapeseed cultivars, the first, more developed, embryos started to be seen after 14 days, in the interspecific hybridisation from the used parental components, the siliquae harvests were mostly late in most of the crosses, as the ovules did not look healthy but yellowish as described by Bhat and Sarla (2004), due to incompatibility barriers and embryo development abortion. It was

stated that, in the used genotypes, interspecifically pollinated ovules remain healthy as long as 10 days after pollination (DAP) and no ovule remain healthy as late as 14 DAP. After observations that embryos can be obtained from earlier harvest i.e. 8 DAP, or that some genotypes have vital ovules at 13 DAP, 11 DAP was establish as the time to obtain the most embryos successfully.

To date, successful attempts at rapeseed resynthesis have been published in numerous scientific papers. However, to the best of our knowledge, the published experiment one was the first study focusing directly on the crossability and hybrid production between diploid winter turnip rapes (as the female components) and diploid winter curly kales.

Regarding the pollination, Bhat and Sarla (2004) examined the causes of variation (i.e. in pollination efficiency and pollen tube entry) during interspecific hybridization, and found important effects of both parental genotypes. Several papers have also reported a visible parental influence on seed and siliquae setting in interspecific crosses (Momotaz *et al.* 1998; Wang & Campbell 1998; Lu *et al.* 2001; Malek *et al.* 2012). Although large differences between mean pollination efficiencies were observed they could not be clearly attributed to the genotypes. A relatively larger spread between the worst and best mean pollination efficiency (46.85–100%, see Table 6) was found in experiment two, which contained fifty crosses against only twenty crosses in the experiment one (50.9%–91.7%), but also no significant effect of a cross combination and no differences between combinations were detected, according to non-parametric Kruskal–Wallis one way analysis of variance and multiple mean comparisons at $P \leq 0.05$. This was most likely due to the rather large differences between replications (see error bars in Fig. 5*a*). Similar results are reported by Bhat and Sarla (2004). We consider that a substantial part of the variability is caused by a combination of physiological differences among states of donor plants during the pollination cycles and bud age (gynoecium immaturity of younger buds), consequentially also the relative siliquae age after harvesting, as well as the emasculation effect as reported by Brown *et al.* (1991). The implicated wounding and damage of the tiny *B. rapa* gynoecium/ovary may also occur during emasculation and bud pollination. For the latter reason, the use of *B. oleracea*, with (on average) bigger buds and ovaries as a female component, seems to be a more feasible option (Brown *et al.* 1991). Another source of variability might be connected with the number of pollinated ovules within individual

siliquae. For example, in our experiments, even the siliquae with only one pollinated ovule were considered "fully pollinated", as it was difficult to accurately distinguish between undeveloped, already pollinated, and non-pollinated ovules during routine isolation after eleven days of pollination. The substantial effect of a genotype on embryo yield has been observed in a number of articles (Lu *et al.* 2001; Bhat & Sarla 2004; Malek *et al.* 2012; Wen *et al.* 2008). Regarding our results according to the statistical analyses, no significant impact of genotype was observed when expressed as the number of embryos per siliqua (i.e. adjusted for the effect of pollination efficiency) or per bud. We assume that such influence was unsteady due to inconsistency among replications (for similar reasons as that for the pollination efficiency). Furthermore, when comparing the variety, in experiment one, where the male components were curly kales, had lower pollination rate than in experiment three, where the selected male components were cabbages with crossability ranging from 70% to 91% (Table 7). Thus, we can estimate that the influence if any will be logically more notable between groups of varieties of parental compontents.

On average, 0.41 embryos/siliquae and 0.32 embryos/bud, respectively were obtained per single bud in fifty different combinations, including the non-productive crosses, as well as the most productive cross, Svalöfs Duro × Kapral, with 2.66 embryos per bud in the best replication. The performance of combinations with zero production of embryos may have been caused by some post-fertilization barriers or genetic incompatibility of these combinations, which were not solvable by means of the embryo rescue method and/or sexual hybridization (Wang *et al.* 2013), or they may be solvable by optimization and larger amount of crosses and different earlier time of harvest. Further studies will be necessary to confirm any of these hypotheses. Nevertheless, we were able to identify several combinations, which were among those with the highest yields. In experiment three, Ante × Vysocké AIK, with 77 embryos was the most productive cross combination using a cabbage component. Good results were achieved in most of the other cross combinations (see Tab. 7). It can be stated that the success of the method in terms of both crossability and embryo production rate were confirmed in such combinations where for the male component were used two genotypes of cabbage, and as a female component, spring and winter turnip rapes. Such hybrid siliquae ratio and hybrid production rate was comparable to the most successful combinations in the experiment one, where for the male component were used curly kales, and as a female component, winter turnip rapes. In general, the technique used with these culture media was appropriate to obtain embryos in

most of the evaluated combinations (fourty-two of fifty) and therefore it is considered suitable for using a large number of different genotypes to produce resynthesized hybrids of oilseed rape.

In a review by Inomata (1990), articles were reported with HPR mean rates from 0.0004 to 0.0786 with *B. rapa* as the female component. In later experiments, Ozminkowski and Jourdan (1994) obtained 0.03, Lu *et al.* (2001) 0.247 HPR, and Wen *et al.* (2008) 0.087 HSR. Our experiments showed good rates of hybrid embryo production via ovule culture through a simplified, less laborious, and more efficient method compared to previously published results (Ozminkowski & Jourdan 1994; Lu *et al.* 2001; Rahman 2004; Rahman 2005; Bennett *et al.* 2008). Improvement of this technique has been highly desired, mainly for the smaller and less robust *B. rapa* as the female parental component because it is generally noted as time-consuming and difficult for small-seeded poly-ovulate species (Reed *et al.* 2005). Avoided ovule counting and one-by-one ovule manipulations resulted in less labour and time requirements; indirectly, permitting taking advantage of most possible materials without excessive ovule drying. Moreover, the procedure implemented (understood as superficial damage at the blade cut, and pulling out of the siliqua contents) may be beneficial, as ovule perforations can increase water and nutrient uptake (Reed *et al.* 2005). Additionally, it is important to mention the fact that successful germination of embryos was achieved in all but one of the crosses out of twenty-four in experiment one and it fourty two of fifty, in total. Happstadius *et al.* (2003) obtained embryos in seventeen out of twenty-four female *B. oleracea* accessions crossed with two *B. rapa* genotypes. Lu *et al.* (2001), Zhang *et al.* (2004), and Wen *et al.* (2008), who all used *B. rapa* as the female parent, reported nine out of twelve, three out of three, and five successful combinations out of five, respectively. These papers represent results with succesful RS rapeseed embryo production, having more than two accessions.

Significant and positive correlations ($r = 0.50^*$ and $r = 0.26^*$) were found between the pollination efficiency and the mean number of embryos per siliqua and per bud. These results could be explained by the fact that crosses producing more embryos per single siliqua, formed more ovules per ovary, and/or exhibited a better ovule pollination ability; regardless of whether it was a matter of genotype, environment, or replication error. Thus, in general, siliquae (genotypes) forming a higher number of pollinated ovules have the potential to be more productive (Bhat & Sarla 2004).

Hybrid morphological characteristics of true leaves were more perceptible after transfer to *in vivo* in the larger and more developed plants (see Fig. 7*d-g*). This probably was caused due to poorer plant development under *in vitro* conditions, and less efficient mesophyll differentiation compared to plants grown in greenhouse conditions (Hazarika 2006). The limitations of such assessments, mentioned above, indicated that reliable identification of hybrid plants at very early developmental stages are clearly needed (Carloni *et al.* 2014).

Flow cytometry can be a valuable tool in the identification of hybrids (Eeckhaut *et al.* 2005; Nakamura *et al.* 2005; Benavente *et al.* 2008). In previously published studies, FCM analyses have been used for RS oilseed rape hybrid verification (Ozminkowski & Jourdan 1994; Heath & Earle 1995; 1997; Sosnowska *et al.* 2010). However, only in our study, did precise and detailed histograms clearly distinguish the parental components and hybrids (see Fig. 6). We consider that it was possible primarily due to large differences in nuclear DNA content among all the parental cultivars used (Table 5). It has been stated that divergence in genome size between the parental components should reach at least 6–8% in order to reliably distinguish hybrids via FCM analyses (Suda *et al.* 2007). Concerning *B. rapa* and *B. oleracea*, Johnston *et al.* (2005) observed an average difference of about 31%, allowing us to use bulked samples composed of up to 5 regenerants for all parental combinations. As a result, the number of analyses was reduced by five times, similar to that described by both Schranz *et al.* (2005), and Krejcíkova *et al.* (2013). Thus, the usage of bulked samples contributed greatly to the reduction of the time and costs without compromising the reliability of the method; further, it proved to be an excellent tool for detection of hybrid regenerants at early stages of *in vitro* regeneration. We expect FCM (with the use of bulked samples) to be generally applicable for the detection of resynthesized genotypes. This applies also in the case of reciprocal crosses and/or in other *Brassica* species, as long as there exists a sufficient distance between genome sizes of the respective parents. Using FCM for early detection of hybrids would be even more desirable in cases using low contrast parents, where morphological evaluations may not be applicable. Although we were also able to detect the hybridity of regenerants by means of cytological analyses, the whole procedure was evaluated as being time consuming and disadvantageous compared to FCM analyses (Takahira *et al.* 2011). In addition, it was laborious to reliably distinguish between cells with twenty chromosomes and nineteen chromosomes (compare Fig. 7*a* and 7*b*) when analysing larger numbers of samples.

In the next experiments, it was also corroborated that due to highly significant differences in relative DNA content between all hybrid combinations and their respective parental components it was possible to reliably assess the hybrid nature of all regenerants via flow cytometry. As the occurrence of the self-pollinated and/or somatic-tissue regenerated female parent was not detected, the hybridity of all regenerants was reliably verified. Morphological assessment of regenerated plants showed typical characteristics originating from both parental components, and further corroborated the results of flow cytometric analysis.

Flow cytometric analyses also revealed signs of endopolyploidy in curly kale (see relevant peaks in Fig. 6*b-d*) and in hybrid samples (Fig. 6*c, e*), a commonly occurring phenomenon typical for certain plant families, including Brassicaceae (Jovtchev *et al.* 2007). Thus, respective peaks in the case of bulked samples (see Fig. 6*e*) may have represented endopolyploidy and/or spontaneous doubled haploids, commonly seen during gametophytic embryogenesis in *B. napus* (Kudo & Kimura 2001; Takahira *et al.* 2011; Gu *et al.* 2014). Therefore, possible overlapping peaks in the case of bulked samples may complicate the identification of spontaneous doubled haploids. Nevertheless, we theorize a very low diploid rate, if any; and this would be desired even for further work with the plant materials (Ferrie & Möllers 2011).

Herein it has been presented a relatively simple, fast, and efficient procedure for the production of hybrid, properly-developed embryos through the embryo rescue technique, without contamination of self-pollinated and/or somatic-tissue-regenerated female components. Moreover, combinations that exhibited high embryo yields, and positive correlations between the pollination efficiency (HSR and HPR) were detected. Through the maximizing of resynthesized embryo yield, the acquisition of the desired number of embryos can be achieved with fewer siliquae; therefore, further shortening, simplifying, and reducing the cost of the whole procedure. Since there were selected, for the crossings, materials with cold resistance (mostly winter type vegetables), they are thought to bring cold-resistant genes, among other characteristics. In general, the produced materials and the applied methods are considered useful in breeding programmes aimed at diversifying the winter oilseed rape gene pool.

In experiment three, it was dealed with ensuring the fertility of the resynthesized rapes, after a rutine diploidization protocol, with colchicine, at the Crop Research Institute,

in order to obtain fertile flowers by chromosome doubling and seed set in a wide rage of crosses, which is essential in the breeding process. On average, 51% of colchicine treated regenerants formed flower stalks with fertile flowers; and after self-pollination of flowers or flower buds, respectively cross-breeding of bud with fertile rape pollen, only 38% of these plants set seeds. The results confirmed the applicability of the used chromosome doubling protocol via colchicine treatment after *in vitro* conditions for the production of fertile plants with seed set and it was applied in the previously obtained combinations of resynthesized rape (from earlier experiments).

Regarding the plant viability and seed set after colchicination, seeds from self pollinated RS plants were more difficult to obtain (low yield) compared to their progenitors and conventional rapeseed cultivars. Nevertheless, in addition to different polyploidization effectivity (diploidization rate), normal side effects involved in an antimitotic treatment (aneuploidy, chimeras with flower stalks of different ploidy) reduced the possibility to obtain higher seed yield. The low seed set/fertility could be explained by poor RS lines development (Jesske *et al.* 2013) and meiotic irregulatities in genome stability with fertility problems due to the restructuring of merged genomes (Szadkowski *et al.* 2010; 2011) or genetically controlled sterility (Karim *et al.* 2014). The latter refers to a certain intraspecific autoincompatibility of the parental components, which could influence at the interspecific level (Lu *et al.* 2001). These characteristics were observed in the cultivated material. Thus, some mechanism may apply for both the intra and interspecific level (Lu *et al.* 2001), for example, autoincompatibility and high or low seed set of *B. rapa* and *B. oleracea*, will effect on the respective RS offspring (*B. napus*). Therefore, resynthesized rapes can acquire strong genes for autoincompatibility and other seed set characteristics from its predecesors (Lu *et al.* 2001; Karim *et al.* 2014). On the other hand, the process of resynthesis itself means genomic instability and rearrengements due to a fusion of two haploid unpairing genomes with reversions, homoelogous nonreciprocal transpositions, new allele recombinations, (de)methylations, (de)acetylations, etc which evoke newly transposomes and genes (de)activation, patterns of chromosome pairing, the effect of extra gene dosage, gene expression orchestration and concerted DNA replication different from the initially separated genetic resources (Feldman & Levy 2005; Gaeta *et al.* 2007). Resynthetized rape, as a two genomes fusion, gives rise to genetic disbalances including possible chromosome losses (Wang *et al.* 2008). Therefore, additional extra

genetic instability is pronounced in the case of *B. napus* resynthesis (Gaeta *et al.* 2007). This is a general phenomenom reported in hybridizations giving rise to allopolyploidy as it creates a considerable stress on the plant, in the way that allopolyploidization triggers rapid genome changes (revolutionary changes) through the instantaneous generation of a variety of cardinal genetic and epigenetic alterations (Feldman & Levy 2005). Furthermore, Guo *et al.* (2016) presented, that after many years of work with over 120 conserved RS lines, despite most of them were genetically stabilized, few are still in segregation. This may complicate characterization and multiplication of RS genotype lines. On the other hand, it can be also considered as evolutionary genome changes facilitated by allopolyploidy, which promote by mostly genetic changes, genetic diversity flexibility and adaptability, to points that are not attainable at the diploid level and can be achieved by further hybridization of natural and synthetic allopolyploids (Feldman & Levy 2005).

Furthermore, plant tissue cultures itself during the process of embryo rescue represent a shock which can induce further somatoclonar variability and epigenetic changes. The latter have different stability, some of them may be permanent or only a number of generations, depending on external factors, and some of them even pass to the next generation. In addition to embryo recue *in vitro* cultivation; colchicination adds further stress (Dr. Ladislav Kučera - personal communication). Thus, induced mutations and somatoclonar variability may occur after application of antimitotic agents in the process of diploidization. It is possible that plant materials, taken from each auto(in)compatible cultivars, when combined and taken to *in vitro* culture and their plantlets then taken to green house conditions (new change, new stress), can be influenced at the epigenetic or mutagenic level even activating or deactivating genes which led to different auto(in)compatibily than the starting materials or the final output. Therefore, within the first generation it is difficult to claim wherther the plant is autocompatible or not because it is, first of all, a matter of stress, genomic rearrangement, viability and different seed set (Gaeta *et al.* 2007, Lu *et al.* 2001). Then, it is a matter of context, here; autoincompatibily can not be separated from fertility, absolutely. Thus, we can not definitely classify the autoincompatibility within only one generation which possibly will further segregate and have a quite untraditionally stressed background.

In the family brassicaceae, for example, C genome from conventional *B. napus* is not homologue to the C genome of *B. oleracea* vegetables. Therefore, once we get an

autopollinised RS rape (which is not a uniform line, as these newly combinations of genomes are not stable and cleave i.e. in Guo *et al.* (2016)), its cross with a conventional rape can compromise further genomic stabilization between the resynthesized rape and the conventional rape. In such crosses, as there is not linear paring, instability of the genome and meiotic disorders and block-locked locuses with limited crossing overs due to different chromosomal lenghts, translocations, inversions, deletions, etc account to genomic changes. Such chromosome changes in RS rapeseed can also corretale with reduced seed yield and reduced pollen viability (Xiong *et al.* 2011). Another side effect from the phenomenon of genome fusion could be the nucleolar dominance of the chromosomes nucleolar organizer regions (NOR) or other NOR changes (Xiong *et al.* 2011). It was found that in leaves of RS *B. napus*, unlike the situation in natural *B. napus*, the nucleolar dominance of *B. rapa* over *B. oleracea* ribosomal RNA genes was incomplete suggesting that genotype differences among the progenitors of natural and synthetic *B. napus* may affect the extent of *B. oleracea* ribosomal RNA gene expression (Chen & Pikkard 1997).

Regarding the obtained seed materials, the seed quality of RS materials (including their parental components) and their crosses with conventional/elite varieties of winter oilseed rape were tested by comparing seed quality parameters through near infrared spectroscopy and gas chromatography. The heredity effect of some traits was viewed in experiment four with NIRS analyses on seed quality parameters. Both female parental components of the RS combinations were selected for these tests because of their known good quality parameters of high oleic acid content and none of erucid acid. The RS material from those genotypes crossed with one regular cabbage (with expected poor seed quality) resulted in consistent proof of poor seed quality parameters after resynthesis. Althought both female parental genotypes presented very similar values, their cross with the same cabbage, had different quality parameters. Therefore, while the genotypes used as female component had similar seed quality parameters, its genetic backgrounds proved to have a great influence in the quality parameters of the resulting hybrids due to the distinct interactions within the parental component genotypes. Among these two hybrids, characteristic of the RS genotype "Antvys" (“Ante”x “Vysocké”) was a lower proportion of erucic acid while characteristic of the RS genotype "Jumvys" (“Jumbuck”x “Vysocké”) was higher oiliness and higher erucic acid content.

When crossed to donors of quality, the cross of “Antvys” with“Cadeli” contributed to a notably reduction in the erucid acid content and glucosinolates (to ~ 5%, respectively, 49 umol/g) and a slight increase in oleic acid content (48%). The cross of “Jumvys”x “Ladoga” had a less pronounced effect on the reduction of erucic acid and glucosinolates (to ~ 32%, resp. 65 umol) and the increase of oleic acid content (32%). In the latter combination, the oiliness did not improve compared to its RS alone but it was higher than in Jumbuck, the RS female component (34%, 35% and 32%, respectively). In “Antvys”x “Cadeli” the oiliness, despite it was higher than in the RS alone, was the same as in Ante, the RS female component (35%, 32% and 35%, respectively). Therefore, in either case, the cross with the quality donor failed to significantly increase the oiliness of the crossing.

In general, these results indicate that although it wasn’t possible to find a clear relationship within the seed parameters of the RS combination and its parental components, the influence of the parental components may vary according to the crossed combination. Furthermore, these preliminary results suggest the importance of appropriate combinations of "RS rape × donor" both in terms of the rate of improvement and the overall quality of the resulting F_1 hybrid. Moreover, it seems to be two approaches; on one hand, to cross different RS materials with one quality donor cultivar, and obtain diferent quality parameters, on the other hand, for an specific RS cultivar with and specific trait, to be crossed with different elite donor genotypes in order to comparatively test which quality donor genotype maximizes the improvement in quality parameters, before subsequently backcrossing aimed to keep a RS desire trait with elite seed quality. However, such procedures could be shortened and be more efective with the help of microspore cultures crossed with one embryogenic elite cultivar, for the fast introduction into breeding programs.

F_1 hybrids of RS rapes crossed with the variety Ladoga as the male component, in experiment five, did not show a dramatic reduction of the erucic acid content, similarly as in experiment four. Because it was also here recorded a negative correlation with the oleic acid content, its percentual increase was only partial, in one case it content even decreased. This is consistent with the results of the screening done using Near-Infrared Spectroscopy (NIRS), where the used donors of quality were Ladoga and Cadeli (chapter 6.2.4 / Hilgert *et al.* 2015b). Unlike in the RS rapes, a negative correlation was found between linoleic acid, and eicosenoic acid and no relationship between the aforementioned acids and α-

linolenic acid. On average, there was a slight increase in the content of linoleic acid, a slight decline in linoleic acid and no changes with eicosenoic acid. A very interesting result was seen in the genotype VODVYS, with high α-linolenic acid contrast, where by contrast to the other RS genotypes, its F_1 hybrid with Ladoga showed a significant increase in this acid (from 11.0 to 16.1%, Ladoga 7.7%).

It was not proved the assumption that the inclusion of turnip rapeseeds with a high-quality oil into the resynthesis crosses as parent components will transfer to the appropriate extent, their quality parameters in the resulting RS rapes. i.e. using spring turnip rape Ante and Jumbuck with undetectable erucic acid content acid and high oleic content (59.0 resp. 53.2%) after resynthesis the cabbage cv. Vysocký AIK, which shows among the used paternal components a relatively high oleic acid content and low erucic acid content, results in RS rapes, approaching more its male component with poorer oleic acid content and erucic acid content still high above the threshold (Tab. 9).

Another assumption, which was not proved, was that RS rapes with poor oil quality and rape conventional varieties with high oil quality will already give rise to a significant improvement of the fatty acid profiles in the first crossed generation. For oleic acid content, it was, at best, improved by one third of the original percentage. For erucid acid content, it was, in two cases, reduced to about half of the original percentace (ANTVYS, VODVYS), similarly as to the previously published results of crossing ANTVYS with the donor quality Cadeli (chapter 6.2.4 / Hilgert *et al.* 2015b). Still, its percentual content is still high above the accepted threshold.

On the other hand, they were obtained RS rapes with higher percentage of α-linolenic acid (12-14%), than in conventional rapeseed varieties. It was also very interesting the significant increase of α-linolenic acid content obtained after crossing VODVYS with Ladoga, where similar values were not as high in either of the parental component; similar results were achieved in Lu *et al.* (2001), where in one RS rape fatty acid profile, the concentration of α-linolenic acid was 9.8%, the conventional oilseed used contained 6.2% and the resulting F_1 hybrid reached a 18.5%.

In summary, from presented results, resynthesized (RS) materials can be a potentially a good source of specific fatty acids (FA) profiles in the oil, which could be transferred by crossing to conventional rapeseeds. Besides the increased content of

desirable fatty acids (i.e. oleic, linoleic, and α-linolenic acids), it is more likely and also expected a higher content of antinutritional substances (erucid acid and glucosinolates). Based on analyzes of fatty acid profiles of seven RS genotypes, it can be stated that, in addition to the increased α-linolenic acid content obtained in few of them, there were not found further specific profiles with significant contents of other important and / or other minor fatty acids. Apart from the known relationship between the content of oleic acid and erucic acid, linoleic and α-linolenic acids, it was not found a general relationship, according to which it would be possible, on the basis of the spectrum of fatty acid profiles of the starting components, to estimate the resulting RS rape profile. These results indicate that the influence of the starting components for the oil quality of RS genotypes, by crossing with conventional varieties can be ambiguous and variable. Nevertheless, further selection from this material via microspore culture establishments could provide promising material for the use of resynthesis in breeding programmes.

In order to obtain new specific fatty acid profiles and to expand rapeseed diversity in this area, it will be therefore necessary either to significantly expand the gene pool with RS material or use methods of mutagenesis and transgenesis. On the other hand, by suitable combinations of parental components and subsequent crossing with conventional varieties it should be possible to maximize, respectively, partially settle desired fatty acid contents, respectively; reduce the anti-nutritional fatty acid contents in the early stages of breeding.

Seed set of the RS × L crosses showed in experiment six was far poorer and uncomparable with the productive donor of quality Ladoga. Nevertheless, the resulting cross of RS with this donor of quality had better seed set than the respective RS (more consistant, as many diploid RS plants did not seed set or seed set ocationally). As the RS materials used as female components had genetically inestable backgrounds, their cross with Ladoga performed also variable seed set in each plant. It is difficult to define their autocompatibility with only this experiment for the reasons explained earlier (pages 71-73). Further genetic changes and genetic diversity may be then conditioned with the cross of such synthetic fusion, as it is the resynthesis of oilseed rape, with the breed cultivars. Further selfing and split of material will be necessary, but we can state that two combinations from the material used were productive in selfing and their seeds should perform the same autocompatibility (Buzel × L and Grusty × L). Interestingly, this

happened in these to combinations despite all used RS material as female components are autoincompatible, according to previous observations: i.e. Bulharská × Kadeřávek Zelený, which can be selfed only after pollinisation in bud, once it was crossed with Ladoga produced in technical isolation a clearly autocompatible hybrid. Nevertheless, an additional effect was observed, in individual cases, that apart from autoimcompatibility of used cultivars, the crossability and seet sed were likely affected due to other genetic problems. Similar results of wide range of seed set (1- 22 seeds per siliquae) and different crossability were presented by Lu *et al.* (2001). The RS with Ladoga crosses were, in the best cases, comparable in siliquae length (7 cm) with normal oilseed rape cultivars (see Fig. 12).

Regarding microspore cultures, the protocol used was aimed to produce DiHaploid lines. Embryogenic lines via microspore culture can help to shorten the breeding cycles. Only four of six used variants produced embryos from viable microspores. The size of the bud correlates with the ideal phase of the microspores for the induction of embryos in the microspore culture protocol. Independently of the variety in rape, the ideal bud size can vary even among plants of one variety cultivated under same conditions.The ideal bud sizes for all used the materials for the microspore cultures were within the range of normal oilseed rape; for our experiments aroung 3 mm. The combinations Grusty × L and Grusca × L produced the most and least number of embryos, respectively.

The genotype is a very important factor for the embryogenic potencial, and, as Ladoga is embryogenic and it is observed that commonly the male component is the donor of embryogenicity. We can support this hypothesis as more than half of the hybrids were embryogenic, but also that in RS material, genetic instability can play a key role, independently of the genotypes used: it was noticed that the morphology in some plants, and the seed set showed some abnormalities or problems with flowering among individual of the same crosses. Furthermore, despite the morphological selection of the best plants, observations in the microscopes cultures support this hypothesis as Arkpet × L microspores had an atypical square shape and formed clusters, and the donor plants from these microspores seed set only ocasionally. On the other hand, it is worth mentioning that also the process of creating two homologous chromosomes from the haploid microspore culture can spontaneously induce further inbreeding problems and genetic disbalances. Nevertheless, seed set of this hybrid cross in some of other plants from the same harvested seeds was unexpectably better (data not shown). In few more lines, similar inconsistency

among plants of the same cross in cultivation after this experiment, was seen, where independetly of the cross, time and plant donors used, some of the plants, set long siliques sporadically in technical isolation, while others seed set nothing.

The material here created via microspore cultures is a source of diversity in which it will be possible to select seed quality parameters from Ladoga in combination to other desired parameters from the RS genotypes in addition to general genetic diversity, i.e. cold tolerance. The produced material was numbered and the cotyledon leaves were extracted and frost in a non-destructively way. Once the dihaploid lines will be selfed, the seeds and respective cotyledons will be analyse in order to find a correlation in seed quality for early selection. According to the amount of obtained seeds from individual DH lines the method chosen will be: for larger quantities of seeds (from 0.8 g) near infrared spectroscopy (NIRS) and for small amount of seeds, gas chromatography (GC), optimized for minimum weight of the sample according to Endlová *et al.* (2014), in collaboration with the research and development OSEVA Ltd. Opava. GC method will be used especially in cases of limited quantities of seeds, due to auto-incompatibility genotypes and therefore, the need to perform bud pollination. Selected parameters of nutritional quality of seeds DH regenerants will be analyzed and compared with similar characteristics, parental components using STATISTICA.

To summarize this chapter, it was helpful to work on *B. napus* embryos needs and development to approach and optimize an embryo rescue protocol. In the first and second experiment, it was demonstrated a reliable protocol to resynthesize oilseed rape from its equivalent ancestors with a simple technique avoiding individual ovule count, excesive handling time which in consequence could let dry and kill the unprotected embryos and/or ovules. A reliable early verification of the RS embryos with bulked samples was also developed in collaboration of the co-authors of this experiment. In the second and third experiment, it is confirmed the effectivity of this techniques in a wide range of genotypes for breeding programs. It was also corroborated the effectiveness of the resynthesis protocol including information on hybrid plantlet regeneration all the way to fertile plants via colchicine treatment. In the fourth and fift experiment, the seed quality was studied. In the sixth experiment, the RS lines crossability with conventional rapeseeds and the embryogenic potential of the obtained crosses was analysed.

8 CONCLUSIONS

It can be concluded, that:

1. Oilseed rape, fertile and usable in breeding programs can be obtained by crossing selected genotypes of *B. rapa* and *B. oleracea*, with the combination of optimized ovule culture with early verification and diploidization methods. The above procedure of oilseed rape resynthesis is sufficient enough to be applicable in breeding programmes aimed at diversity expansion of winter oilseed rape gene pool, as the resynthesized embryos were derived in most combinations.

2. Clear differences were found in the hybrid production rates of the used combinations. It is assumed that this was in part due to the specific genomic compatibility of the starting components, as specific combinations, and not only due to each particular physiological or genetic genotype characteristics.

3. Winter oilseed rape type, requiring vernalisation for the transition to the generative state, can be resynthesized by crossing selected genotypes of winter turnip rape and kale or cabbage. Vernalisation was partially needed or had at least a positive effect in crosses of spring turnip rape with cabbage (except in the case of "Jumvys" (“Jumbuck”x “Vysocké”) and "Antvys" (“Ante”x “Vysocké”), which behaviour as spring cultivars).

4. All produced embryos were true hybrids. The obtained diploidization rates were comparable to rates published in scientific literature. The diploidization of the created material by colchicine treatment also involved chimeric plants and/or diploid plants with damaged fertility. The latter happened also problably due to genetic instability caused by spontaneous uncontrolled DNA rearrengements proper of the interspecific cross.

Fertile regenerants can be self-pollinated and crossed with elite commercial lines and the autoincompatibility of starting cultivars can be overcome.

5. Resynthesized rapes from selected genotypes of *B. rapa* and *B. oleracea* genotypes can be obtained with poor seed quality but specific fatty acids oil content (i.e. higher linolenic acid content) and other unique features absent in the available range of varieties of rapeseed.

6. The created RS lines presented poor seed quality parameters from one or both starting components. After crossing RS material with quality donors, all monitored parameters showed only a slight improvement. However, specific fatty acid profiles were more contrasting in certain crosses and it is expected to select interesting quality combinations after further breeding.

7. Embryogennic material aimed to stablish microspore cultures can be created from crosses of RS material with quality donors when the quality donors are used as male components. The produced material from microspore cultures can content specific fatty acid profiles while conserving the original genetic diversity and other desired parameters such as cold tolerance.

BENEFITS AND USES

Obtained plant material (resynthesised genotypes, their crosses with donors of quality and DH lines) in the form of seeds and regenerants *in vitro*, acquired and developed during this dissertation thesis will be used as a unique source material in breeding programs, and will be available as a model material for further study as well.

The published articles in peer-reviewed journals can serve as a basic source of information for further study, optimization and application of published procedures not only within the genus *Brasssica*, but also will be applicable for other members of the family Brassicaceae.

OUTLOOK

In addition to seed quality parameters, research using the created material will be aimed to identify other promising signs, useful in breeding of rape, as autoincompatibility, resistance to diseases, pests, etc.

Further work will focus on the relationship between frost resistance / susceptibility, respectively, winter hardiness from starting components and rapeseeds, resynthetized from winter turnip rape and kales, and rapeseeds, resynthesized from winter turnip rapes and cabbage as well as their comparison with european linear varieties. Also the vernalisation needed for rapeseeds, resynthesized from spring turnip rapes with cabbages will be stablished for its routine inclusion in the breeding process.

Crosses of perspective materials were crossed in order to obtain new sources of yellow-seeded *Brassica napus*. New optimization for some of these combinations was out of the scope of this thesis. The resulting hybrids are succesfully cultivated *in vitro* conditions and it is expected to regenerate fertile plants and study their properties by next year within the The Czech National Project QJ1510172 - The use of non-conventional source materials, biotechnological methods and effective procedures in line and hybrid breeding of winter oilseed rape (2015-2018, MZE/QJ).

Further research is also aimed at evaluation of various antimitotic agents in terms of their efficiency to double chromosome number and obtained regenerants.

9 REFERENCES

Adamska A, Cegielska-Taras T, Kaczmarek Z, Szala L (2004) Multivariate approach to evaluating the fatty acid composition of seed oil in a doubled haploid population of winter oilseed rape (*Brassica napus* L.). J Appl Genet 45:419–425

Akbar MA (1989) Resynthesis of *Brassica napus* aiming for improved earliness and carried out by different approaches. Hereditas (Lund) 111:239–246

Appelquist LA, Ohlson R (1972) Rapeseed Cultivation. Composition, Processing and Utilization. Elsevier publishing Company

Banga SS, Labana KS (1991) Cytoplasmic-genetic relationship between *Brassica nigra* and *Sinapis allioni*. Cruciferae Newsletter 14/15: 12–13

Beckman C (2005) Vegetable oils: competition in a changing market. Bi-weekly Bulletin. Agriculture and Agri-Food Canada 18(11)

Benavente E, Cifuentes M, Dusautoir JC, David J (2008) The use of cytogenetic tools for studies in the crop-to-wild gene transfer scenario. Cytogenet Genome Res 120:384–395

Bennett RA, Thiagarajah MR, King JR, Rahman MH (2008) Interspecific cross of *Brassica oleracea* var. *alboglabra* and *B. napus*: effects of growth condition and silique age on the efficiency of hybrid production, and inheritance of erucic acid in the self-pollinated backcross generation. Euphytica 164:593–601

Bhojwani SS, Razdan M.K (1996) Plant Tissue Culture: Theory and Practice (Elsevier, Amsterdam) 125–166

Bhat S, Sarla N (2004) Identification and overcoming barriers between *Brassica rapa L.* em. Metzg. and *B. nigra* (L) Koch crosses for the resynthesis of *B. juncea* (L) Czern. Genet Resour Crop Ev 51:455–469

Brown AP, Brown J, Dyer AF (1991) Optimal pollination conditions for seed set after a self-pollination, an intraspecific cross and an interspecific cross of marrow-stem kale (*Brassica oleracea* var. *acepala*). Euphytica 51:207–214

Budak H, Shearman RC, Parmaksiz I, Gaussoin RE, Riordan TP, Dweikat I (2004) Molecular characterization of buffalograss germplasm using sequence related amplified polymorphism markers. Theor Appl Genet 108:328–334

Campion B, Perri E, Azzimonti MT, Vicini E, Schiavi M (1995) Spontaneous and induced chromosome doubling in gynogenic lines of onion (*Allium cepa* L.). Plant Breed 114:243–246

Carloni E, Ribotta A, López-Colomba E, Griffa S, Quiroga M, Tommasino E, Grunberg K (2014) Somatic embryogenesis from *in vitro* anther culture of apomictic buffel grass genotypes and analysis of regenerated plants using flow cytometry Plant Cell Tiss Organ Cult. doi: 10.1007/s11240-014-0441-4

Carlos J, Dias S (1999) Effect of activated charcoal on *Brassica oleracea* microspore culture embryogenesis. Euphytica 108:65–69

Chalhoub B, Denoeud F, Liu S, Parkin IAP, Tang H, Wang X, Chiquet J, Belcram H, Tong C, Samans B, Correa M, Da Silva C, Just J, Falentin C, Koh CS, Le Clainche I, Bernard M, Bento P, Noel B, Labadie K, Alberti A, Charles M, Arnaud D, Guo H, Daviaud C, Alamery S, Jabbari K, Zhao M, Edger PP, Chelaifa H, Tack D, Lassalle G, Mestiri I, Schnel N, Le Paslier MC, Fan G, Renault V, Bayer PE, Golicz AA, Manoli S, Lee TH, Thi VHD, Chalabi S, Hu Q, Fan C, Tollenaere R, Lu Y, Battail C, Shen J, Sidebottom CHD, Wang X, Canaguier A, Chauveau A, Berard A, Deniot G, Guan M, Liu Z, Sun F, Lim YP, Lyons E, Town CD, Bancroft I, Wang X, Meng J, Ma J, Pires JC, King GJ, Brunel D, Delourme R, Renard M, Aury JM, Adams KL, Batley J, Snowdon RJ, Tost J, Edwards D, Zhou Y, Hua W, Sharpe AG, Paterson AH, Guan C, Wincker P (2014) Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. Science 345:950–953

Chatterjee D, Banga S, Gupta M, Bharti S, Salisbury PA, Banga SS (2016) Resynthesis of *Brassica napus* through hybridization between *B. juncea* and *B. carinata*. Theor Appl Genet 129 5:977-990

Chen BY, Heneen WK (1989) Resynthesized *Brassica napus* L.: A review of its potential in breeding and genetic analysis. Hereditas 111:255–263

Chen ZJ, Pikaard CS (1997) Transcriptional analysis of nucleolar dominance in polyploid plants: Biased expression/silencing of progenitor rrRNA genes is developmentally regulated in *Brassica.* Proc Natl Acad Sci USA 94:3442–3447

Choudhary BR, Joshi P (2000) Cytomorphology of intergeneric hybrid *Sinapis alba·x Brassica nigra*. J Genet Breed 54:157–160

Coventry J, Kott L, Beversdorf WD (1988) Manual for Microspore Culture Technique for *Brassica napus*. University of Guelph.

Cui C, Ge HX, Gautam M, Kang L, Li ZY (2012) Cytoplasmic and Genomic Effects on Meiotic Pairing in *Brassica* Hybrids and Allotetraploids from Pair Crosses of Three Cultivated Diploids. Genetics 191:725–738

Delourme R, Brun H, Ermel M, Lucas MO, Vallee P, Domin C, Walton G, Li Hua, Sivasithamparam K, Barbetti MJ (2008) Expression of resistance to *Leptosphaeria maculans* in *Brassica napus* double haploid lines in France and Australia is influenced by location. Ann Appl Biol 153:259–269

Dhooghe E, Van Laere K, Eeckhaut T, Leus, L, Van Huylenbroeck J (2011) Mitotic chromosome doubling of plant tissues *in vitro*. Plant Cell Tiss Org 104:359–373

Dixon GR (2007) Vegetable Brassicas and Related Crucifers Crop production science in horticulture; 14. ISBN: 978 0 85199 395 9

Dolezel J, Greilhuber J, Suda J (2007) Estimation of nuclear DNA content in plants using flow cytometry. Nat Protocols 2:2233–2244

Duijs JG, Voorrips RE, Visser DL, Custers JBM (1992) Microspore culture is successful in most crop types of *Brassica oleracea* L. Euphytica 60 45–55

Eeckhaut T, Leus L, Van Huylenbroeck J (2005) Exploitation of flow cytometry for plant breeding. Acta Physiol Plant 27:743–750

Endlová L, Vrbovský V, Pastrňák J (2013) Minimalizační postupy stanovení mastných kyselin v řepce. *Úroda* 12/2013, vědecká příloha časopisu

Endlová L, Vrbovský V, Klíma M, Navrátilová Z (2014) Hodnocení skladby mastných kyselin v dělohách mikrosporových embryí řepky olejky (*Brassica napus*). Úroda 12/2014, vědecká příloha časopisu

Evigez (2013) Plant genetic resources documentation in the Czech Republic. (http://genbank.vurv.cz/genetic/resources/). Accessed 30 August 2013

Feldman M, Levy AA (2005) Allopolyploidy—a shaping force in the evolution of wheat genomes. Cytogenet Genome Res 109:250–258

Ferrie AMR, Keller WA (2004) *Brassica* improvement through microspore culture. In: Pua EC, Douglas CJ (eds.): Biotechnology in Agriculture and Forestry. *Brassica*. Springer Verlag, Berlin, 54:149–168

Ferrie AMR, Möllers C (2011) Haploids and doubled haploids in *Brassica* spp. for genetic and genomic research. Plant Cell Tiss Organ Cult 104:375–386

Ferrie AMR, Palmer CE, Keller WA (1995) Haploid embryogenesis. In: Thorpeta TA (ed): *In vitro* Embryogenesis in Plants. Kluwer, Dordrecht, 309–344

Formanova N, Li XQ, Ferrie AMR, DePauw M, Keller WA, Landry B, Brown GG (2006) Towards positional cloning in *Brassica napus*: generation and analysis of doubled

haploid *B. rapa* possessing the *B. napus* pol CMS and Rfp nuclear restorer gene. Plant Mol Biol 61:269–281

FitzJohn RG, Armstrong TT, Newstrom-Lloyd LE, Wilton AD, Cochrane M (2007) Hybridisation within *Brassica* and allied genera: valuation of potential for transgene escape. Euphytica 158:209–230

Gaeta RT, Pires JC, Iniguez-Luy F, Leon E, Osborn TC (2007) Genomic changes in resynthesized *Brassica napus* and their effect on gene expression and phenotype. Plant Cell 19: 3403–3417

Geoffriau E, Kahane R, Bellamy C, Rancillac M (1997) Ploidy stability and *in vitro* chromosome doubling in gynogenic clones of onion (*Allium cepa* L.). Plant Sci 122:201–208

Girke A, Schierholt A, Becker HC (2012) Extending the rapeseed genepool with resynthesized *Brassica napus* L. I: Genetic diversity. Genet Resour Crop Ev 59:1441–1447

Gómez-Campo C (1999) Developments in Plant Genetics and Breeding 4, Biology of *Brassica* Coenospecies. Elsevier

Gómez KA, Gómez AA (1984) Statistical procedures for agricultural research, 2nd edition. John Wiley and Sons, New York, ISBN 0-471-87092-7

Grzebelus E, Adamus A (2004) Effect of anti-mitotic agents on development and genome doubling of gynogenic onion (*Allium cepa* L.) embryos. Plant Sci 167:569–574

Gu HH, Zhao ZQ, Sheng XG, Yu HF, Wang JS (2014) Efficient doubled haploid production in microspore culture of loose-curd cauliflower (*Brassica oleracea* var. *botrytis*). Euphytica 195:467–475

Guo S, Zhang X, Zeng D, Zhang M, Niu Y, Wang L, Liu Y (2016) Genetic diversity of resynthesized *Brassica napus* lines from SW China assessed by main agronomic traits and SSR markers in comparison with common B. napus lines. Euphytica 207 1:95-108

Gupta SK (Ed) (2013) Biotechnology of crucifers 2013 Springer-Verlag Gmbh ISBN: 9781461477945

Hansen NJP, Andersen SB (1996) *In vitro* chromosome doubling potential of colchicine, oryzalin, trifluralin, and APM in *Brassica napus* microspore culture. Euphytica, 88:156–164

Heath DW, Earle ED (1995) Synthesis of high erucic acid rapeseed (*Brassica napus* L.) somatic hybrids with improved agronomic characters. Theor Appl Genet 91:1129–1136

Heath DW, Earle ED (1996) Resynthesis or rapeseed (*Brassica napus* L): A comparison of sexual versus somatic hybridisation. Plant Breed 115:395–401

Heath DW, Earle ED (1997) Synthesis of low linolenic acid rapeseed (*Brassica* napus) through protoplast fusion. Euphytica 93:339–343

Hemmingway JS (1976) Mustards: *Brassica* spp. and *Sinapis* alba. In: NW Simmonds (Ed.), Evolution of Crop Plants. Longman Inc. NY

Hilgert A, Klíma M, Urban M (2014) Efektivní resyntéza řepky pomocí techniky Ovule culture. Úroda 62, 12/2014, vědecká příloha časopisu.

Hilgert-Delgado A, Klíma M, Viehmannová I, Urban MO, Fernández-Cusimamani E, Vyvadilová M (2015a) An Effective Method Of Resynthesized Oilseed Rape Embryo Production Via Ovule Culture Of Different Crosses Between Winter Turnip Rape And Curly Kale. Plant Cell Tiss Orga 120:191-201

Hilgert-Delgado A, Vrbovský V, Endlová L, Klíma M (2015b) Parametry kvality semen u vybraných resyntetizovaných řepek a donorů kvality stanovené pomocí NIRS Sborník abstraktů 14. Konference experimentální biologie rostlin P10–1: 92

Hilgert-Delgado A, Vrbovský V, Endlová L, Urban MO, Klíma M (2015c) Tvorba nových genotypů a hodnocení vybraných parametrů kvality semene resyntetizované řepky. Úroda 63, 12/2015, vědecká příloha časopisu

Inomata N (1990) Interspecific hybridization in *Brassica* through ovary culture. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, Legumes and oilseed crops I. Springer, Berlin Heidelberg New York Tokyo, 10:367–384

Inomata N (1991) Intergeneric hybridisation in *Brassica juncea* × *Sinapis pubescens* and *B. napus* × *S. pubescens*, and their cytological studies. Cruciferae Newsl 14/15 :10–11

Inomata N (1992) Intergeneric hybridisation between *Brassica carinata* and *Sinapis turgida* through ovary culture, and cytology and crossability of their progenies. Genet (Life Sci Adv) 1 l:129–140

Inomata N (1994) Intergeneric hybridisation between *Brassica napu*s and *Sinapis pubescens*, and the cytology and crossability of their progenies. Theor Appl Genet 89:540–544

Jakse M, Havey MJ, Bohanec B (2003) Chromosome doubling procedures of onion (*Allium cepa* L.) gynogenic embryos. Plant Cell Rep 21:905–910

Jesske T, Olberg B, Schierholt A Becker HC (2013) Resynthesized lines from domesticated and wild *Brassica* taxa and their hybrids with *B. napus* L.: genetic diversity and hybrid yield. Theor App Genet 126: 1053–1065

Johnston JS, Pepper AE, Hall AE, Chen ZJ, Hodnett G, Drabek J, Lopez R, Price HJ (2005) Evolution of genome size in *Brassica*ceae. Annal Bot 95:229–235

Kershaw MT (1998) Ethnobotanical Leaflets / Southern Illinois University Carbondale / http://opensiuc.lib.siu.edu/cgi/viewcontent.cgi?article=1385&context=ebl

Karim MM, Siddika A, Tonu NN, Hossain DM, Meah MB, Kawanabe T, Fujimoto R, Okazaki K (2014) Production of high yield short duration *Brassica napus* by interspecific hybridization between *B. oleracea* and *B. rapa*. Breeding Sci 63:495–502

Klíma M, Vyvadilová M, Kucera V (2004) Production and utilization of doubled haploids in *Brassica oleracea* vegetables. Hortic Sci (Prague) 31:119–123

Klíma M, Vyvadilová M, Kučera V (2008) Chromosome doubling effects of selected antimitotic agents in *Brassica napus* microspore culture. Czech J Genet Plant Breed 44:30–36

Klíma M, Abraha E, Vyvadílová M, Bechyně M (2009) Protoplast culture and fusion between *Brassica carinata* and *Brassica napus*. *Agricultura tropica et subtropica* 42(1) 34-45.

Krejcíková J, Sudová R, Lucanová M, Trávnícek P, Urfus T, Vít P, Weiss-Schneeweiss H, Kolano B, Oberlander K, Dreyer LL, Suda J (2013) High ploidy diversity and distinct patterns of cytotype distribution in a widespread species of Oxalis in the Greater Cape Floristic Region. Ann Bot 111:641–649

Kudo N, Kimura Y (2001) Flow cytometric evidence for endopolyploidy in seedlings of some *Brassica* species. Theor Appl Genet 102:104–110

Kuginuki Y, Miyajima T, Masuda H, Ken-Ichi Hida, Hirai M (1999) Highly regenerative cultivars in microspore culture in *Brassica oleracea* L. var. *capitata*. Breed Sci 49:251–256

Li M, Chen X, Meng J (2006) Intersubgenomic heterosis in rapeseed production with a partial new-typed *Brassica napus* containing subgenome A^r from *B. rapa* and C^c from *Brassica carinata*. Crop Sci 46: 234–242

Li M, Qian W, Meng J, Li Z (2004) Construction of novel *Brassica napus* genotypes through chromosomal substitution and elimination using interploid species hybridisation. Chromosome Res 12:417–426

Lichter R (1982) Induction of haploid plants from isolated pollen of *Brassica napus*. Zeitschrift für Pflanzenphysiologie 105:427–434

Lichter R (1989) Efficient yield of embryoids by culture of isolated microspores of different *Brassicae* species Plant Breed 103:119–123

Liu S, Wang H, Zhang J, Fitt BDL, Xu Z, Evans N, Liu Y,Yang W, Guo X (2005) *In vitro*mutation and selection of doubled-haploid *Brassica napus* lines with improved resistance to *S. sclerotiorum*, Plant Cell Rep 24:133–144

Lu CM, Zhang B, Kakihara F, Kato M (2001) Introgression of genes into cultivated *Brassica napus* through resynthesis of *B. napus* via ovule culture and the accompanying change in fatty acid composition. Plant Breeding 120:405–410

Lysak MA, Dolezel J (1998) Estimation of nuclear DNA content in *Sesleria* (Poaceae). Caryologia 52:123–132

Malek MA, Ismail MR, Rafii MY, Rahman M (2012) Synthetic *Brassica napus* L.: development and studies on morphological characters, yield attributes, and yield. The Scientific World Journal [online]. doi: 10.1100/2012/416901

Mohapatra D, Bajaj YPS (1987) Interspecific hybridisation in *Brassica juncea* × *Brassica hirta* using embryo rescue. Euphytica 36:321–326

Möllers C, Iqbal MCM Röbbelen (1994) Efficient production of doubled haploid *Brassica* napus plants by colchicine treatment of microspores. Euphytica 75:95–104

Möllers C, Rucker B, Schierholta A (2000) *In vitro* selection for oleic and linoleic acid content in segregating populations of microspore derived embryos of *Brassica napus*. Euphytica 112: 195–201

Momotaz A, Kato M, Kakihara F (1998) Production of intergeneric hybrids between *Brassica* and *Sinapis* species by means of embryo rescue technique. Euphytica 103:123–130

Morejohn LC, Bureau TE, Molè-Bajer J, Bajer AS, Fosket DE (1987) Oryzalin, a dinitroaniline herbicide, binds to plant tubulin and inhibits microtubule polymerisation *in vitro*. Planta 172:252–264

Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nakamura T, Kuwayama S, Tanaka S, Oomiya T, Saito H, Nakano M (2005) Production of intergeneric hybrid plants between *Sandersonia aurantiaca* and *Gloriosa rothschildiana* via ovule culture (Colchicaceae). Euphytica 142:283–289

Nath UK, Goswami G, Clemens R, Becker HC, Möllers C (2009) Inheritance and variation of erucic acid content in a transgenic rapeseed (*Brassica napus* L.) doubled haploid population. Mol Breeding 23:125–138

Navabi ZK, Parkin IAP, Pires JC, Xiong Z, Thiagarajah MR, Good AG, Rahman MH (2010) Introgression of B-genome chromosomes in a doubled haploid population of *Brassica napus* × *B. carinata*. Genome 53:619–629

Navrátilová B (2004) Protoplast cultures and protoplast fusion focused on *Brassica*ceae—a review. Hort Sci (Prague) 31:140–157

Olsson G (1960) Species Crosses Within The Genus *Brassica*. 2. Artificial *Brassica napus* L. Hereditas 46: 351–386

Odstrcilová L, Plachká E (2005) Significance of *Leptosphaeria maculans* and *Sclerotinia sclerotiorum* incidence in winter rapeseed in the Czech Republic. In: Int. Workshop Integrated Control In Oilseed Crops. Inst Plant Genet Poznan

Ozminkowski RH, Jr, Jourdan P (1994) Comparing the resynthesis of *Brassica napus* L. by interspecific somatic and sexual hybridisation. I. Producing and identifying hybrids. J Am Soc Hortic Sci 119:808–815

Pecinka A, Fang W, Rehmsmeier M, Levy AA, Mittelsten Scheid O (2011) Polyploidization increases meiotic recombination frequency in Arabidopsis. BMC Biol 9:24

Polidoro J (2008) Turnips. College Seminar 235 Food for Thought: The Science, Culture, & Politics of Food, Spring 2008

http://academics.hamilton.edu/foodforthought/our_research_files/turnips.pdf

Prakash S, Hinata K (1980) Taxonomy, cytogenetics and origin of crop *Brassicas*, a review. Opera Bot 55:1–57

Prakash S, Wu XM, Bhat SR (2011) History, evolution, and domestication of *Brassica* crops. Plant Breed Rev 35:19–84

Rahman MH (2005) Resynthesis of *Brassica napus* L. for self-incompatibility: self-incompatibility reaction, inheritance and breeding potential. Plant Breed 124:13–19

Rahman MH, Bennett RA, Yang R-C, Thiagarajah MR (2011) Exploitation of the late flowering species *Brassica oleracea* L. for the improvement of earliness in *B. napus* L. – an untraditional approach. Euphytica 177:365–374

Rahman H (2013) Breeding spring canola (*Brassica napus* L.) by the use of exotic germplasm. Can J Plant Sci 93:363–373

Reed SM (2005) Embryo rescue. In: Trigiano RN, Gray DI (eds): Plant Development and Biotechnology. CRC Press, Boca Raton, FL, USA 235–239

Rich TCG (1991) Crucifers of Great Britain and Ireland. Botanical Society of the British Isles, London. ISBN 13:9780901158208

Ripley VL, Arnison PG (1990) Hydridization of *Sinapis alba* L. and *Brassica napus* L. via embryo rescue. Plant Breed 104:26-33.

Rudolf K, Bohanec B, Hansen M (1999) Microspore culture of white cabbage, *Brassica oleracea* var. *capitata* L.: Genetic improvement of non-responsive cultivars and effect of genome doubling agents. Plant Breed 118:237–241

Scarano MT, Tusa N, Abbate L, Lucretti S, Nardi L, Ferrante S (2003) Flow cytometry, SSR and modified AFLP markers for the identification of zygotic plantlets in backcrosses between 'Femminello' lemon cybrids (2n and 4n) and a diploid clone of 'Femminello' lemon (Citrus limon L. Burm. F.) tolerant to mal secco disease. Plant Sci 164:1009–1017

Seyis F, Snowdon RJ, Luhs W, Friedt W (2003) Molecular characterization of novel resynthesized rapeseed (*Brassica napus*) lines and analysis of their genetic diversity in comparison with spring rapeseed cultivars. Plant Breed 122:473–478

Seyis F (2013) Towards a Canola Quality Resynthesized Rapeseed (*B. napus* L.) *B. olaracea* Genotypes as A Basic Resource. European Journal of Plant Science and Biotechnology 7 (Special Issue 1) 33–37

Schranz ME, Dobes C, Koch MA, Mitchell-Olds T (2005) Sexual reproduction, hybridisation, apomixis, and polyploidization in the genus Boechera (*Brassica*ceae). Am J Bot 92:1797–1810

Song K, Tang K, Osborn TC (1993) Development Of Synthetic *Brassica* Amphidiploids By Reciprocal Hybridisation And Comparison To Natural Amphidiploids. Theor Appl Genet 86:811–821

Smýkalová I, Vetrovcová M, Klíma M, Macháčková I, Griga M (2006) Efficiency of Microspore Culture for Doubled Haploid Production in the Breeding Project "Czech Winter Rape". Czech J Genet Plant 42:58–71

Sosnowska K, Szala L, Olejnik A, Cegielska-Taras T (2010) Wstepne badania nad resynteza rzepaku ozimego (*Brassica napus* L.) – Preliminary study on resynthesis of winter oilseed rape (*Brassica napus* L.). Roślliny oleiste – Oilseed Crops Tom XXXI 257–265

Sosnowska K, Cegielska-Taras T (2014) Application of *in vitro* pollination of opened ovaries to obtain *Brassica oleracea* L. × *B. rapa* L. hybrids. In Vitro Cell Dev-Pl 50:257–262

Springer B, Wojciechowski A (2006) Ocena efektywnosci kultur *in vitro* izolowanych zarodków i zalazków w otrzymywaniu mieszanców oddalonych w rodzaju *Brassica* – The efficiency of obtaining interspecific hybrids in *Brassica* genera with applying *in vitro* culture of isolated embryos and ovules. Roślliny oleiste – Oilseed Crops Tom XXVII 187–196

Sridevi O, Sarla N (1996) Reciprocal hybridisation between *Sinapis* alba and *Brassica* species. Cruciferae Newsletter 18:16

Suchý P, Straková E, Herzig I (2008) Kvalita rostlinných olejů a jejich význam z hlediska zdraví zvířat a možnosti ovlivnění nutriční hodnoty potravin živočišného původu. Vědecký výbor výživy zvířat Prague, VUZV.

Suda J, Kron P, Husband BC, Trávnícek P (2007) Flow cytometry and ploidy: applications in plant systematics, ecology and evolutionary biology. In: Dolezel J, Greilhuber J, Suda J (eds.): Flow Cytometry with Plant Cells. Analysis of Genes, Chromosomes and Genomes 103–130. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

Szadkowski E, Eber F, Huteau V, Lodé M, CoritonO, Jenczewski E, Chèvre AM (2011) Polyploid formation pathways have an impact on genetic rearrangements in resynthesized *Brassica* napus. New phytol 191:884–894

Szadkowski EEF, Huteau V, Lodé M, Huneau C, Belcram H, Coriton O, Manzanares-Dauleux MJ, Delourme R, King GJ, Chalhoub B *et al.* (2010) The first meiosis of resynthesized *Brassica napus*, a genome blender. New Phytol 186: 102–112

Szala L, Sosnowska K, Poplawska W, Liersch A, Olejnik A, Kozlowska K, Bocianowski J, Cegielska-Taras (2016) Development of new restorer lines for CMS *ogura*

system with the use of resanthesized oilseed rape (*Brassica napus* L.). Breeding sci *in press* http://doi.org/10.1270/jsbbs.15042

Takahira J, Cousin A, Nelson MN, Cowling WA (2011) Improvement in efficiency of microspore culture to produce doubled haploid canola (*Brassica napus* L.) by flow cytometry. Plant Cell Tiss Organ 104:51–59

U N (1935) Genome analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. Jpn J Bot 7:389–452

Van Tuyl JM, De Jeu MJ (1997) Methods for overcoming interspecific crossing barriers. In: Shivanna KR, Sawhney VK (eds): Pollen biotechnology for crop production and improvement. Cambridge University Press 273–292

Vyvadilová M, Zelenková S (1992) Responsiveness in microspore cultures of some cultivars and Czech breeding materials of rapeseed (*Brassica napus* L.). Czech J. Genet. Plant Breed 28:243–252

Vyvadilová M, Kucera V, Tomásková D (1998a) Embryogenesis in isolated microspore cultures in different genotypes of *Brassica oleracea*. Zahradnictví Hort Sci (Prague), 25:9–14

Vyvadilová M, Klíma M, Kučera V (1998b) Analysis of factors affecting embryogenesis in microspore cultures of some cruciferous vegetables. Zahradnictví - Hort Sci (Prague), 25:137–144

Wang J, Jiang JJ, Wang YP (2013) Protoplast fusion for crop improvement and breeding in China. Plant Cell Tiss Org 112:131–142

Wang M, Zhao J, Peng Z, Guo W, Wang Y, Wang L, Xia G (2008) Chromosomes are eliminated in the symmetric fusion between *Arabidopsis thaliana* L. and *Bupleurum scorzonerifolium* Willd. Plant Cell Tiss Org 92:121–130

Wang YJ, Campbell C (1998) Interspecific hybridisation in buckwheat among *Fagopyrum esculentum, F. homotropicum,* and *F. tataricum*. Proc. 7th Intl Symp Buckwheat at Winnipeg, Canada 1–13

Wang J, Jiang JJ, Wang YP (2013) Protoplast fusion for crop improvement and breeding in China. Plant Cell Tiss Org, 112:131–142

Warwick SI (1993) Guide to the wild germplasm of *Brassica* and allied crops. Part IV. Wild species in the tribe Brassiceae (Cruciferae) as sources of agronomic traits. Centre for Land and Biological Resources Research, Research Branch, Agriculture Canada, p 1–19

Warwick SI, Francis A, Al-Shehbaz IA (2006) *Brassica*ceae: species checklist and database on CD-ROM. Pl Syst Evol 259:249–258

Warwick SI, Francis A, Gugel RK (2009) Guide to Wild Germplasm of *Brassica* and Allied Crops (tribe Brassiceae, *Brassica*ceae) 3rd Edition

Weiss, E.A., Oilseed Crops 1983, Longman Group Limited, pg. 161-215

Wen J, Tu J-X, Li Z-Y, Fu T-D, Ma C-Z, Shen J-X (2008) Improving ovary and embryo culture techniques for efficient resynthesis of *Brassica napus* from reciprocal crosses between yellow-seeded diploids *B. rapa* and *B. oleracea*. Euphytica 162:81–89

Wojciechowski A (1985a) Interspecific hybrids between *Brassica campestris* L. and *B. oleracea* L. I. Effectiveness of crossing, pollen tube growth, embryogenesis. Genet Pol 26:423–436

Wojciechowski A (1985b) Interspecific Hybrids Between *Brassica* campestris L and *Brassica oleracea* L .2. Morphological Traits - Somatic Chromosome-Number - Meiotic Division and Microsporogenesis. Genet Pol 26:437–446

Yadav P, Bhat SR, Prakash S, Mishra LC, Chopra VL (2009) Resynthesized *Brassica juncea* lines with novel organellar genome constitution obtained through protoplast fusion. J Genet 88: 109–112

Yemets AI, Blume YB (2008) Progress in plant polyploidization based on antimicrotubular drugs. Open Hort J 1:15–20

Xiao Y, Chen L, Zou J, Tian E, Xia W, Meng J (2010) Development of a population for substantial new type *Brassica napus* diversified at both *A*/*C* genomes. Theor Appl Genet 121:1141–1150

Wu J, Li F, Xu K, Gao G, Chen B, Yan G, Wang N, Qiao J, Li J, Li H *et al.* (2014) Assessing and broadening genetic diversity of a rapeseed germplasm collection. Breed Sci 64: 321–330

Xiong Z, Gaeta RT, Pires JC (2011) Homoeologous shuffling and chromosome compensation maintain genome balance in resynthesized allopolyploid *Brassica napus.* PNAS 2011 108 (19) 7908-7913

Xu Y, Xu H, Wu X, Fang X, Wang J (2012) Genetic changes following hybridisation and genome doubling in synthetic *Brassica napus*. Biochem Genet 50:616–624

Zhao J, Simmonds DH (1995) Application of trifluralin to embryogenic microspore cultures to generate doubled haploid plants in *Brassica napus*. Physiologia Plantarum 95:304–309

Zhang GQ, Tang GX, Song WJ, Zhou WJ (2004) Resynthesizing *Brassica napus* from interspecific hybridisation between *Brassica rapa* and *B. oleracea* through ovary culture. Euphytica 140:181–187

Zhang GQ, He Y, Xu L, Tang GX, Zhou WJ (2006) Genetic analyses of agronomic and seed quality traits of doubled haploid population in *Brassica napus* through microspore culture. Euphytica 149:169–177

Zhang HZ, Shi CH, Wu JG (2011) Analysis of genetic effects for heterosis of erucic acid and glucosinolate contents in rapeseed (*Brassica napus* L.). Agricultural Sciences in China 10:1525–1531

10. APPENDICES

Apendix A List of Author's Thesis Publications

A1 Publication in Scientific Journal with IF

Hilgert-Delgado A, Klíma M, Viehmannová I, Urban MO, Fernández-Cusimamani E, Vyvadilová M (2015). An Effective Method Of Resynthesized Oilseed Rape Embryo Production Via Ovule Culture Of Different Crosses Between Winter Turnip Rape And Curly Kale. Plant Cell Tiss Orga 120:191-201

A2 Procedings

Hilgert-Delgado A, Vrbovský V, Endlová L, Urban MO, Klíma M (2015): Tvorba nových genotypů a hodnocení vybraných parametrů kvality semene resyntetizované řepky. Úroda 63 12/2015, vědecká příloha časopisu

Hilgert A, Klíma M, Urban M (2014) Effective resynthesis of rapeseed via ovule culture technique. Úroda 62, 12/2014, vědecká příloha časopisu

Hilgert-Delgado A, Klíma M, Viehmannová I, Fernández Cusimamani E (2014) Effective Techniques for Resynthesized Rapeseed Production of Contrasting Components via Ovule Culture and Flow Cytometry. Tropentag, 17-19.09, 2014. Czech University of Life Sciences Prague Czech Republic

A3 Conference Abstracts

Hilgert-Delgado A, Vrbovský V, Endlová L, Klíma M. Parametry kvality semen u vybraných resyntetizovaných řepek a donorů kvality stanovené pomocí NIRS. Sborník abstraktů 14. Konference experimentální biologie rostlin 2015: P10 – 1: 92 Oral presentation

Effective Techniques for Resynthesized Rapeseed Production of Contrasting Components via Ovule Culture and Flow Cytometry. Hilgert-Delgado A, Klíma M, Viehmannová I, Fernández Cusimamani E.Tropentag, Prague, Czech Republic September 17-19, 2014

Apendix B A Taxonomic Synopsis of *Brassica's A*llied Genera with Indication of Subgenera, Sections, Species and Subspecies

***ERUCASTRUM* C. Presl.**

E. gallicum O.E.Schulz
E. nasturtiifolium (Poiret) O.E.Schulz subsp. *nasturtiifolium*
E. n. subsp. *sudrei* Vivant
E. leucanthum Coss. and Dur.
E. palustre (Pir.) Vis.
E. virgatum C.Presl. subsp. *virgatum*
E. v. subsp. *baeticum* (Boiss.) Gómez-Campo
E. v. subsp. *brachycarpum* (Rouy) Gómez-Campo
E. v. subsp. *pseudosinapis* (Lange) Gómez-Campo
E. varium Durieu subsp. *varium*
E. v. subsp. *mesatlanticum* Maire and Wilczek
E. v. subsp. *subsiifolium* Maire
E. littoreum (Pau and F.Quer) Maire subsp. *littoreum*
E. L subsp. *brachycarpum* (Maire and Weiller) Gómez-Campo
E. l. subsp. *glabrum* (Maire) Gómez-Campo
E. rifanum (Emb. and Maire) Gómez-Campo
E. elatum (Ball.) O.E.Schulz
E. brevirostre (Maire) Gómez-Campo
E. canariense Webb and Berth.
E. cardaminoides (Webb ex Christ.) O.E.Schulz
E. ifniense Gómez-Campo
E. arabicum Fisch. and Mey.
E. elgonense Jonsell
E. meruense Jonsell
E. abyssinicum (A. Rich.) O.E.Schulz
E. pachypodum (Chiov.) Jonsell
E. rostratum (Balf. f.) Gómez-Campo
E. strigosum (Thunb.) O.E.Schulz
E. griquense (Brown) O.E.Schulz

***DIPLOTAXIS* DC.**

Subgen. *Diplotaxis*

D. tenuifolia (L.) DC. subsp. *tenuifolia*
D. t. subsp. *cretacica* (Kotov) Sobrino-Vesperinas
D. muralis (L.) DC. subsp. *muralis*
D. m. subsp. *ceratophylla* (Batt.) Mart.-Lab.
D. viminea (L.) DC.
D. simplex (Viv.) Spr.

Subgen. *Hesperidium* (DC.) Mart.-Lab.

D. harra (Forsk.) Boiss. subsp. *harra*
D. h. subsp. *confusa* Mart.-Lab.
D. h. subsp. *crassifolia* (Rafin) Maire
D. h. subsp. *lagascana* (DC.) O..Bolós and J.Vigo
D. antoniensis Rustan

D. glauca (Schmidt) O.E.Schulz
D. gorgadensis Rustan subsp. *gorgadensis*
D. g. subsp. *brochmanii* Rustan
D. gracilis (Webb) O.E.Schulz
D. hirta (Chev.) Rustan and Borgen
D. sundingii Rustan
D. varia Rustan
D. vogelii (Webb) Cout.
D. pitardiana Maire
D. acris (Forsk.) Boiss.
D. villosa Boul. and Jail.
D. griffithii (Hook.f. and Thoms.) Boiss.
D. nepalensis Hara

Subgen. *Rhynchocarpum* (Prantl) Mart.-Lab.

Sect. *Rhynchocarpum* Prantl

D. assurgens (Del.) Gren.
D. tenuisiliqua De1. subsp. *tenuisiliqua*
D. t. subsp. *rupestris* (J. Ball.) Mart.-Lab.
D. brachycarpa Godr.
D. virgata (Cav.) DC. subsp. *virgata*
D. v. subsp. *australis* Mart.-Lab.
D. v. subsp. *rivulorum* (Br.-B1. and Maire) Mart.-Lab.
D. v. subsp. *sahariensis* (Coss.) Mart.-Lab.
D. berthautii Br.-B1. and Maire
D. catholica (L.) DC.
D. ollivieri Maire
D. siifolia G. Kunze. subsp. *siifolia*
D. s. subsp. *bipinnatifida* (Coss.) Mart.-Lab.
D. s. subsp. *vicentina* (P.Cout.) Mart.-Lab.

Sect. *Heterocarpum* Mart.-Lab.

D. ibicensis (Pau) Gómez-Campo *D. siettiana* Maire
D. brevisiliqua (Coss.) Mart.-Lab.
D. ilorcitana (Sennen) Aedo and Mart.-Lab.

Sect. *Erucoides* Mart.-Lab.

D. erucoides (L.) DC. subsp. *erucoides*
D. e. subsp. *longisiliqua* (Coss.) Gómez-Campo

***SINAPIS* L.**

Sect. *Sinapis*

S. alba L. subsp. *alba*
S. a. subsp. *mairei* (H.Lindb.) Maire
S. a. subsp. *dissecta* (Lag.) Bonnier
S. flexuosa Poir.

Sect. *Ceratosinapis* DC.

S. arvensis L. subsp. *arvensis*
S. a. subsp. *allioni* (Jacq.) Baillargeon
S. a. subsp. *nilotica* (O.E.Schulz) Baillargeon

Sect. *Hebesinapis* DC.

S. pubescens L. subsp. *pubescens*
S. p. subsp. *virgata* (Batt.) Baillargeon
S. boivinii Baillargeon
S. indurata Coss.
S. aristidis Coss.

Sect. *Chondrosinapis* O.E.Schulz

S. aucheri (Boiss.) O.E.Schulz

ERUCA Mill.

E. vesicaria (L.) Cav. subsp. *vesicaria*
E. v. subsp. *sativa* (Miller) Thell.
E. v. subsp. *pinnatifida* (Desf) Emb.and Maire

***COINCYA* Rouy**

C. richeri (Vill.) Greuter and Burdet
C. wrightii (O.E.Schulz) Stace
C. monensis (L.) Greuter and Burdet subsp. *monensis*
C. m. subsp. *cheiranthos* (Vill.) Aedo, Leadlay and Muñoz-Garm.
C. m. subsp. *nevadensis* (Willk.) Leadlay
C. m. subsp. *orophila* (Franco) Aedo, Leadlay and Muñoz-Garm.
C. m. subsp. *puberula* (Pau) Leadlay
C. transtagana (Cout.) Clem.-Mufioz and Hern.-Bermejo
C. longirostra (Boiss.) Greuter and Burdet
C. rupestris Porta and Rigo subsp. *rupestris*
C. r. subsp. *leptocarpa* (Gonz.-Albo) Leadlay

***RAPHANUS* L.**

R. raphanistrum L. subsp. *raphanistrum*
R. r. subsp. *landra* (DC.) Bonnier and Layens
R. r. subsp. *maritimus* (Sm.) Thell.
R. r. subsp. *microcarpus* (Lange) Thell.
R. r. subsp. *rostratus* (DC.) Thell.
R. sativus L.
S.

***HIRSCHFELDIA* Moench**

H. incana (L.) Lagr&e-Fossat subsp. *incana*
H. i. subsp. *incrassata* (Thell.) G6rnez-Campo

***SINAPIDENDRON* Lowe**

T. angustifolium (DC.) Lowe
S. frutescens (Sol.) Lowe subsp. *frutescens*
S. f subsp. *succulentum* (Lowe) Rustan
S. gymnocalyx (Lowe) Rustan
S. rupestre Lowe
S. sempervivifolium Mnzs.

***TRACHYSTOMA* O.E. Schulz**

T. aphanoneurum (Maire and Weiller) M.and W.
T. balli O.E.Schulz
T. labasii Maire

(Gómez-Campo, 1999)

Apendix C Photographic Documentation

C1.1 Different RS genotypes before vernalisation

C1.2 Different RS genotypes before vernalisation

C2 RS line of winter oilseed turnip Brachina and curly kale Kaderávek Zelený

C3 RS line of spring oilseed turnip Jumbuck and cabagge Vysocké AIK

C4 RS line of winter oilseed turnip Svalöfs Duro and curly kale Kapral

C4 RS line of spring oilseed turnip Evvisa and cabbage Zakamenné

C5 RS line of winter oilseed turnip Brachina and curly kale Frosty

C6 RS line of winter oilseed turnip Izumrudnaja K 193 and curly kale Kapral

C7 RS line of winter oilseed turnip Svalöfs Duro and curly kale Kaderávek Zelený

C7 RS line of winter oilseed turnip Bulharska and curly kale Scarlet

C8 RS line of spring oilseed turnip Jumbuck and cabbage Zakamenné

C9 RS line of turnip rape V17 and cabbage Vysocké AIK

C10 RS line of turnip rape V17 and cabbage Vysocké AIK –flowers detail

C11 Fertile RS line of spring oilseed turnip Ante and cabbage Zakamenné

C12 Detail of trichomes in the RS of the cross of spring oilseed rape Ante and cabbage Vysocké AIK

C13 RS line of winter oilseed turnip Arktus and curly kale Pentland Brig

C14 RS lines isolated for self-pollination or hand pollinised buds and siliquae