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FACULTY OF SCIENCE
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PROTOPLAST CULTURES OF SELECTED MEMBERS OF THE FAMILY BRASSICACEAE

Ph.D. THESIS

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Ph.D. Program of Biology – Botany

Supervisor: RNDr. Božena Navrátilová, Ph.D.

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Abstract:
Protoplast fusion and the regeneration of fusion products in a protoplast culture are used predominantly for manipulation of foreign genes to broaden the gene pool, i.e. to expand genetic diversity. Thus, protoplast culture technique can be emphasized as a perspective suplement in crop improvement programmes, particularly in overcoming crossability barriers through somatic hybridization. Efficient and reliable protocols for protoplast isolation, electrofusion, chemical fusion and protoplast cultivation in selected *Brassica* genotypes have been developed. Proper combinations of concentration and treatment time of polyethylene glycol (PEG) determined protoplast fusion frequency between genotypes used. In general, 25% PEG combined with 20 minutes treatment duration produced satisfactory fusion frequency and good rate of viability. Formation of cell divisions and microcallus structures were detected almost in all genotypes tested. However, strong impact of the genotype on the initialization of cell divisions and further development of dedifferentiated tissue was observed. Microcalli were obtained from chemical fusion between *Brassica rapa* and *B. carinata,* and between *B. carinata* and *B. napus*, whole plants transferred to *in vivo* from protoplast cultures of *Brassica oleracea*. Somatic hybrids with properly developed leaves were obtained and subcultivated *in vitro* after chemical fusion between *B. carinata* and *B. rapa*. The accumulation of cold-induced dehydrin and proline was related to the level of frost tolerance in the protoplast-derived calli of several *Brassica* ssp. cultivars. A ~47kDa dehydrin was detected in the callus tissue of two *B. napus* cultivars. However, the prediction of frost tolerance in *B. napus* and *B. carinata* based on dehydrin or proline detection and quantification in the protoplast-derived calli was not feasible as there was no relationship with levels of these compounds, detected in the leaves.
Keywords: Brassicaceae; *Brassica* ssp.; protoplast isolation; protoplast culture; protoplast fusion; electrofusion; chemical fusion; polyethylene glycol; somatic hybridization; flowcytometry; frost tolerance; proline; dehydrins; one-dimensional electrophoresis; western blot technique.
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DECLARATION

I herewith declare that I autonomously carried out the Ph.D. Thesis entitled "Protoplast cultures of selected members of the family Brassicaceae" during the course of combined doctoral study, led by supervisors RNDr. Božena Navrátilová, Ph.D. and Ing. Vratislav Kučera, CSc., and with the literature that is cited in the Thesis.

In Olomouc .....

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CONTENTS

1. Introduction

The Ph.D. Thesis entitled "Protoplast cultures of selected members of the family Brassicaceae" was prepared under the combined doctoral study program of Biology (field study Botany) at the Department of Botany, Faculty of Science, Palacký University in Olomouc. However, the majority of experiments with the plant material was conducted in laboratories and greenhouses of the Department of Genetics and Plant Breeding Methods, Division of Plant Genetics, Breeding and Product Quality at the Crop Research Institute, Prague. Electrofusion of protoplasts were carried out at the Potato Research Institute in Havlíčkův Brod and some parts of experiments related to chemical fusogens were performed at the Department of Botany, Olomouc.

The topic of my Ph.D. study arose along with the need to introduce new methods of modern biotechnology into breeding of winter oilseed rape and some *Brassica* vegetables at the Crop Research Institute I am working at. One of my workloads is to establish breeding procedures more efficient and to speed up the breeding process of important *Brassica* crops. Somatic hybridization and subsequent regeneration of fusion products represents a unique tool, which can help extend the biodiversity of crops both in qualitative and quantitative traits. By means of protoplast fusion techniques it is possible to create hybrid combinations, which are not feasible via traditional procedures of wide hybridization, whether due to various crossability barriers or the production of nonviable hybrids. Although the latter could be partially overcome by using of embryo rescue techniques, even these methods do not guarantee the vigour and fertility of resulting hybrids.

Hence, research teams of three institutions applied for a joint project comprised protoplast culture and somatic hybridization techniques. Since 2004, the four-year project entitled "Use of protoplast fusion technology in breeding of commercially important crops, *Brassica*, *Cucumis* and *Solanum*" was supported by the Czech Ministry of Agriculture. Concerning *Brassica* crops, in initial experiments we optimised the process of *in vitro* preparation of donor plants, isolation and purification of protoplasts in selected cruciferous genotypes (in term of yield and vitality of isolated protoplasts) and subsequent regeneration from protoplasts in term of cell wall regeneration and first cell division. The improvement of plant regeneration was achieved through changes in media composition.

Consequently, we prepared and further optimised protocols for chemical fusion and protoplast electrofusion. For the chemical fusion, we determined an appropriate combination of "fusogen concentration × time duration" with regard to the viability of fusion products and occurrence of undesirable multifusants. Concerning electrofusion, we achieved acceptable viability of fusants and reduced the incidence of multifusants by determining the appropriate parameters of alternate current (AC) for protoplast align-

ment, followed by direct current pulses to perforate the plasma membrane. Fusion of membranes took place after progressively reduced AC voltage. Viable microcalli, calli and whole plants with proven hybridity were obtained from these experiments. However, the regenerants exhibited the reduced viability and were not able to survive under *in vivo* conditions.

In the final phase of our experiments, we tested the possibility of early *in vitro* selection for cold resistance in the protoplast-derived calli of selected winter and spring oilseed rape (*B. napus*) and Abyssinian mustard (*B. carinata*) genotypes, using proteomic and analytical methods. A ~47kDa dehydrin was detected in the callus tissue of two winter oilseed rape cultivars. However, the prediction of frost tolerance in *B. napus* and *B. carinata* based on dehydrin or proline detection and quantification in the protoplast-derived calli was not feasible as there was no relationship with levels of these compounds, detected in the leaves.

The Thesis is presented in the form of publications in Scientific journals and short communications already published or ready to print. Publications are grouped thematically according to the objectives of the Thesis. In the Appendix section there are photographs documenting the process of somatic hybridization, the isolation and purification of protoplasts, protoplast fusion and regeneration of calli and whole plants from protoplast cultures.

2. Aims of the Thesis

The main aims of the Thesis were to gain knowledge in the field of protoplast culture, to establish and optimise the isolation, chemical fusion and electrofusion of protoplasts and further regeneration in the protoplast culture of selected genotypes of the family Brassicaceae. The next step was to verify the suitability of protoplast culture technique for *in vitro* selection of cold-resistant genotypes and the identification of desired genotypes by means of proteomic analytical methods. The work was focused on the following tasks:

1. To prepare the literature review of the current state of protoplast culture and somatic hybridization in the Brassicaceae family, focusing primarily on the major species of the genus *Brassica*.

2. To establish and optimise the method of protoplast isolation and cultivation in selected *Brassica* genotypes and to regenerate whole plants.

3. To determine the parameters of chemical fusion and electrofusion in selected genotypes and to initialize the regeneration after fusion in protoplast cultures.

4. To validate the *in vitro* selection and identification of cold-resistant genotypes using the protoplast culture technique and proteomic analytical methods.

5. To conclude the results of experiments on protoplast culture, protoplast fusion and *in vitro* selection through a discussion format.

3. Summary Results of the Thesis

3.1. Literature review on protoplast culture and somatic hybridization in the Brassicaceae family

3.1.1. Somatic hybridization and protoplast culture in agricultural crops of the family Brassicaceae – a review (*unpublished*)

Somatic hybridization and protoplast culture in agricultural crops of the family Brassicaceae – a review

PREFACE

Production of new crop varieties with required yield and quality depends on the selection of desirable plants, which is only possible if a wide variation is presented in the base population (Bajaj 1994). However, prolonged, unilaterally oriented breeding has caused substantial narrowing of genetic diversity in many crops (Plucknett *et al.* 1983). Related or distant genera of cultivated crops contain a large reservoir of genes covering a variety of desirable traits. Thus, identification and utilization of this germplasm has great potential for crop improvement (Liu *et al.* 2005).

Although conventional breeding methods could also broaden the diversity by means of wide (distant) hybridization, these procedures often suffer from crossing barriers between parents or various abnormalities of resulting hybrids (van Tuyl and de Jeu 1997). For instance, although McNaughton (1973) obtained hybrids from crosses between *Raphanus sativus* and *Brassica oleracea* ("Raphanobrassica"), these plants exhibited poor fertility and vigour. Malek (2007) reported hybrids between *B. rapa* and *B. nigra,* where all the crosses showed complete pollen sterility with shrivelled, pointed tip, and pale colour anthers and reduced filaments and failed to set siliquae and seeds. Choudhary and Joshi (2001) derived hybrids from crosses *B. tournefortii* × *B. rapa* with low pollen fertility. Moreover, Wang and Luo (1998) reported the abortion of hybrid embryos before the stage of maturity.

Above mentioned difficulties have been partially solved by use of *in vitro* fertilization methods, that are able to overcome pre-fertilization barriers, i.e., by applying pollen to excised ovules on an artificial culture medium (Kranz and Dresselhaus 1996). Several hybrids were produced via the technique of *in vitro* pollination: *B. napus* × × *B. campestris* (Zenkteler *et al.* 1987), *B. napus* × *Diplotaxis tenuifolia*, *B. napus* × × *Moricandia arvensis*, *B. oleracea* × *D. tenuifolia* and *D. tenuifolia* × *B. napus* (Zenkteler 1990).

Great progress on wide hybridization techniques has been achieved after the introduction of *in vitro* embryo rescue procedures, enabling regeneration of hybrid plants [*Sinapis alba* × *B. napus* (Ripley and Arnison 1990), *Eruca sativa* × *B. rapa* (Agnihotri *et al.* 1990), *B. oleracea* var. *alboglabra* × *S. turgida* (Momotaz *et al.* 1998), *B. rapa* × × *B. oleracea* (Heath and Earle 1996), *Lesquerella* spp. (Tomasi *et al.* 2002), *B. juncea* × × *B. napus* (Zhang *et al.* 2003), *B. juncea* × *B. napus* (Iqbal *et al.* 2006), *Erucastrum cardaminoides* × *B. oleracea* var. *alboglabra* (Mohanty *et al.* 2009)]. However,

there are reports that even after using of embryo rescue technology regenerated hybrid plants remained sterile, or with very low pollen fertility (Rao *et al.* 1996, Chandra *et al.* 2004, Inomata 2005).

Consequently, due to mentioned involvements it is difficult to transfer the desirable traits to the cultivated species via conventional breeding. Moreover, other barriers such as polyembryony, female and/or male sterility in some crops further complicate recombination and segregation of desirable traits in subsequent generations. As a result, gene flow from the related or distant genera to the cultivated species is minimized (Liu *et al.* 2005).

Mutations induced by chemicals, radiation or in plant and cell tissue culture (termed somaclonal variation) are the first biotechnological approaches, used to extend the genetic diversity. Since the first report on induced mutation of a gene by Muller in 1927 (Bohman *et al.* 1999) indicated the potential application of mutagenesis for plant improvement, numerous plant mutants have been introduced until now (Larkin 1998). Although somaclonal variation appears to be an important source of genetic variability, the percentage of valuable mutations (analogously to induced mutations) is rather low, changes in the structure of genes are random and unpredictable (Brar and Jain 1998). Moreover, epigenetic, potentially reversible modifications occur more frequently than genetic ones and thus complicate further utilization of such "mutants" in subsequent breeding programmes (Ahuja 1998).

Somatic hybridization and genetic transformation are the two most promising alternatives and/or supplements of sexual hybridization and mutation for gene transfer. They have their own features and one cannot overestimate the function of one technique and underestimate the other (Liu *et al.* 2005). Genetic transformation techniques represent exact, aim-oriented manipulation with single genes at a molecular level. Where interesting genes have been identified and isolated, they can be transferred via genetic transformation techniques, but, for most traits, the genes have not been identified, and somatic hybridization might then be the method of choice (Waara and Glimelius 1995). In addition, presence of selection or reporter markers in transgenic plants may invoke negative impacts on public acceptance of the transgenic products (Liu *et al.* 2005).

CHARACTERIZATION OF THE FAMILY BRASSICACEAE

Brassicaceae (Cruciferae) is a large plant family, consists of 338 genera and 3709 species (Warwick *et al.* 2006). The family is clearly most abundant in the Northern Hemisphere, with the major centre of diversification and endemism in the Irano-Turranian region, where some 150 genera and 900 species are found, and a secondary centre in the Mediterranean region, with more than 110 genera and nearly 630 species. More than 600 species native to North America are distributed primarily in the western United States and northern Mexico. In the Southern Hemisphere, there are 340 species native to South America, 110 species in South Africa, and 114 species in Australia and New Zealand (Al-Shehbaz 1984).

Members of the Brassicaceae family are widely cultivated throughout the world as vegetable crops for human consumption, as condiments and spices for improved flavour of human diets, and as fodder crops for livestock feeding (Downey and Rimmer 1993, Warwick *et al.* 2009). The family includes more than 120 weedy species of local or cosmopolitan distribution as well (Al-Shehbaz 1984), but also many ornamentals, mainly from the genera *Erysimum, Iberis, Linnaeus*, *Lobularia, Malcolmia,* and *Matthiola* (Zhou *et al.* 2001). Nevertheless, the largest cultivation of its members is for edible vegetable oil production (Warwick *et al.* 2009). Thus, Cruciferae has been considered one of the ten most economically important plant families (Rich 1991).

The majority of genera (250) are oligotypic with five or fewer species, and 138 of these are monotypic. However, more than half of the species of the family belong to 12 large genera: *Draba* (340), *Erysimum* (180), *Cardamine* (175), *Lepidium* (175), *Alyssum* (170), *Arabis* (170), *Sisymbrium* (90), *Lesquerella* (80), *Rorippa* (75), *Thlaspi* (75), *Heliophila* (72), and *Hesperis* (60). Nevertheless, economically the most important one is the genus *Brassica,* containing 37 different species (Rakow 2004). Those of particular importance are: *Brassica napus*, *B. rapa*, and *B. juncea* as sources of rapeseed oil, and *B. oleracea* as cole crops. The genera *Raphanus* and *Sinapis* are also of major importance, the former cultivated for its edible roots and the latter as a source of mustard condiments along with *B. nigra. Crambe* is cultivated as industrial oil, and the leaves of other genera (e.g. *Eruca* and *Diplotaxis*) are eaten as salad greens (Warwick *et al.* 2009).

In addition to agriculturally important species, there are many wild relatives that have potential as sources of specific oil composition, condiments and other products. Additionally, these relatives could serve as sources of other useful traits, e.g. cytoplasmic male sterility (CMS) for the development of hybrid seed production systems in *Brassica* crop plants or provide the gene pool of resistance to various abiotic and biotic stresses (Guil-Guerrero *et al.* 1999, Rakow 2004, Wang *et al.* 2006, Prakash and Bhat 2007, Warwick *et al.* 2009). Overview of specific traits, available in selected field crops and their wild relatives from the family Brassicaceae, is shown in Table 1.

Table 1: Traits, available in selected crops and their wild relatives from the family Brassicaceae

Traits	Source	References
Cold tolerance	*Descurainia sophia*	Laroche *et al.* 1992
	Thellungiella salsuginea	Griffith *et al.* 2007
	Thlaspi arvense	Zhou *et al.* 2007
Drought tolerance	*Boechera stricta*	Haugen *et al.* 2008
	Brassica tournefortii	Prakash and Bhat 2007
	Diplotaxis harra	Prakash and Bhat 2007
	Lesquerella fendleri	Ploschuk *et al.* 2001
	Morricandia arvensis	McVetty *et al.* 1989
Salt tolerance	*Lepidium densiflorum*	Orsini *et al.* 2010
	Lesquerella fendleri	Dierig *et al.* 2004
Heavy metals hyperaccumulation	*Allysum* ssp.	Ghaderian *et al.* 2007
	Arabidopsis thaliana	Weber *et al.* 2004
	Arabis alpina	Bovet *et al.* 2006
	Capsella bursa-pastoris	Aksoy *et al.* 1999
	Hesperis persica	Krämer 2010
	Lepidium sativum	Robinson *et al.* 2003
	Thlaspi caerulescens	Lombi *et al.* 2001
Disease resistance		
Albugo candida	*Brassica maurorum*	Chrungu *et al.* 1999
Alternaria ssp.	*Brassica elongata*	Siemens 2002
	Camelina sativa	Westman *et al.* 1999
	Capsella bursa-pastoris	Pedras *et al.* 2003
	Diplotaxis ssp.	Klewer *et al.* 2003
Leptosphaeria maculans (Phoma lingam)	*Brassica carinata*	Rimmer and van den Berg 1992
	Camelina sativa	Li *et al.* 2005
	Sinapis arvensis	Siemens 2002
	Thlaspi arvense	Pedras *et al.* 2003
Plasmodiophora brassicae	*Arabidopsis thaliana*	Rehn *et al.* 2004
	Armoracia rusticana	Prakash and Bhat 2007
	Capsella bursa-pastoris	Siemens 2002
	Raphanus sativus	Murakami *et al.* 2000
Sclerotinia sclerotiorum	*Capsella bursa-pastoris*	Chen *et al.* 2007
Verticilium ssp.	*Armoracia rusticana*	Atibalentja and Eastburn 1998
	Brassica rapa	Rygulla *et al.* 2007
Xanthomonas campestris	*Brassica carinata*	Griffiths and Nickels 2001
	Matthiola incana	Westman and Dickson 1998

Traits	Source	References
Photosystem C3-C4	*Diplotaxis tenuifolia*	Bang *et al.* 2003
	Moricandia nitens	Yan *et al.* 1999
Cytoplasmic male sterility	*Brassica napus* (Polima, Shaan 2A)	Wang *et al.* 2002
	Brassica tournefortii (Tournefortii)	Pahwa *et al.* 2004
	Diplotaxis berthautii	Bhat *et al.* 2008
	Enarthrocarpus lyratus	Janeja *et al.* 2003
	Erucastrum canariense	Prakash *et al.* 2001
	Lesquerella fendleri	Dierig and Ray 2009
	Raphanus sativus (Ogura)	Ogura 1968
	Moricandia arvensis (Moricandia)	Bhat *et al.* 2005
	Trachystoma ballii (Trachystoma)	Kirti *et al.* 1997
Self-incompatibility	*Brassica* ssp.	Conner *et al.* 1998
High erucic acid	*Brassica rupestris*	Velasco *et al.* 1998
	Eruca sativa	Yaniv *et al.* 1998
	Erucastrum cardaminoides	Prakash and Bhat 2007
	Sinapis arvensis	Daun *et al.* 2003
High linoleic acid	*Brassica elongata*	Velasco *et al.* 1998
	Orychophragmus violaceus	Prakash and Bhat 2007
High linolenic acid	*Camelina sativa*	Zubr and Matthäus 2002
	Descurainia sophia	Luo *et al.* 1997
	Lepidium sativum	Prakash and Bhat 2007
	Mathiola incana	Ecker *et al.* 1992
Flavonoids	*Arabidopsis* ssp.	Graham 1998
	Camelina sativa	Onyilagha *et al.* 2003
	Thlaspi arvense	Onyilagha *et al.* 2003
High glucosinolates	*Camelina sativa*	Matthäus and Angelini 2005
	Capsella bursa-pastoris	Vaughn and Berhow 2005
	Diplotaxis tenuifolia	Bennett *et al.* 2007
	Eruca vesicaria ssp. *sativa*	Bennett *et al.* 2007
	Kremeriella cordylocarpus	Agerbirk *et al.* 2008
	Lesquerella fendleri	Vaughn and Berhow 2005
	Sinapis ssp.	Vaughn and Berhow 2005
	Schivereckia doerfleri	Goffman *et al.* 1998
Tocopherols	*Isatis* ssp.	Bagci and Özçelik 2009
	Schivereckia doerfleri	Goffman *et al.* 1998

PROTOPLAST CULTURE AND SOMATIC HYBRIDIZATION

1. Introduction

The term protoplast has been defined by Vasil (1976) as a naked plant cell surrounded by plasma membrane and is potentially capable of cell wall regeneration, growth and division. Thus, isolated protoplast *per se* represents a unique system for ultrastructural, genetical, and physiological investigations, i.e. on the structure and function of cell organelles, cytoplasmic membrane transport in plants and cell wall formation (Navrátilová 2004).

Thanks to their ability to take up macromolecules other than DNA, protoplasts are an excellent model in studies of endocytosis at the plasma membrane, and virus uptake and replication in plant cells. Controlled lyses permits the isolation of cellular fractions, including membranes, organelles and vacuoles, the latter being used to study accumulation of compounds, such as sugars. Patch-clamp techniques, similar to those developed for animal cells, have been used to investigate ion transport through the plasma membrane and regulation of the osmotic balance of cells, to assay the longer-term effects of pharmaceuticals, food additives, agrochemicals etc. (Davey *et al.* 2005).

For example, Robertson and Earle (1987) introduced a qualitative assay for the detection of photosynthetic activity in protoplasts of oilseed rape (*Brassica napus*), Rigó *et al.* (2008) studied the localization of GFP-tagged proteins in the protoplast suspension of *Arabidopsis thaliana*, Skagen and Iversen (1995) made series of experiments on the cortical microtubule array in protoplasts from *B. napus.* Effects of novel proteins on the plasma membrane of *B. napus* protoplasts were investigated by Qui *et al.* (1995), the production of polyamines by Papadakis *et al.* (2005) and the interaction of tachyzoites of *Toxoplasma gondii* with *B. napus* protoplasts by Werk and Fischer (1982). The location, physiological status and the viability of mitochondria by means of rhodamine staining examined Wu (1987) in cabbage and cauliflower protoplasts; Kato *et al.* (2010) investigated membrane proteins in protoplasts of A. *thaliana.*

Although protoplasts provide a naked cell system that is equivalent to cultured animal cells but, unlike the latter, exhibiting the unique property of totipotency (Davey *et al.* 2005). This phenomenon has enabled their utilization in agricultural research, among others for various modifications via experimental mutagenesis (Gebhardt *et al.* 1981, Sidorov *et al.* 1981, Mukherjee and Sengupta 1986), genetic transformations and, foremost, through somatic hybridization (Bajaj 1974, Hegazi and Matsubara 1992, Hansen and Earle 1994b).

Concerning genetic transformation, protoplasts of *B. oleracea* var. *botrytis* (cauliflower) were successfully transformed using polyethylene glycol (PEG) in the experi-

ments of Radchuk *et al.* (2002), Nugent *et al.* (2006) and Jiang *et al.* (2010), or by means of *Agrobacterium*-mediated transformation in *B. carinata* (Abyssinian mustard) (Ohlsson and Eriksson 1988) or *B. napus* (Thomzik and Hain 1990, Wang *et al.* 2005) protoplasts. Unfortunately, the genetic basis of many economically important traits such as disease resistance, stress tolerance, yield increase, etc. are largely unknown, and these traits might be subjected to complex regulatory mechanisms which will complicate the molecular approaches to clarify their genetic basis (Hinnisdaels *et al.* 1994).

On the other hand, gene transfer by somatic hybridization can be successful even though the gene and the gene product are unknown or when several genes control the desired character (Hansen 1998). Thus, unlike the manipulation of single genes through genetic transformations, somatic hybridization does not require the identification and isolation of specific genes (Glimelius *et al.* 1991, Waara and Glimelius 1995, Forsberg 1998).

A plant breeder usually obtains hybrids by using various conventional techniques, but the difficulty arises with the sexually incompatible crosses (see Preface). Additionally, while in the sexual hybridization the male parent contributes only (or almost only) nuclear genes and the female parent contributes both nuclear genes and cytoplasm (Hinnisdaels *et al.* 1994), no such unilateral exclusion of cytoplasm exists in the somatic hybridization (Ahuja 1982).

Somatic hybridization, generally defined as a fusion of two distantly related, to closely related plant protoplasts with subsequent regeneration of a hybrid plant, brings together three genetic systems – the nuclear, mitochondrial and chloroplast genomes of two genetically different and potentially sexually incompatible parents (Eberhard 1980). As a result, a single somatic hybridization event can generate more genetic variation than sexual hybridization (Yadav *et al.* 2009).

Somatic hybridization has several potential advantages over sexual hybridization:

— broadening the gene pool by exchange of genetic material beyond the limits of sexual compatibility (Hu *et al.* 1999, Liu *et al.* 2005, Deng *et al.* 2007) via the transfer of useful traits from wild relatives to phylogenetically remote crop plants (Craig and Millam 1995, Waara and Glimelius 1995, Sigareva and Earle 1999, Sakhno *et al.* 2007, Scholze *et al.* 2010)

— the transfer of traits, controlled via cytoplasmic genes, such as male sterility, herbicide resistance, photosynthesis (Kirti *et al.* 1991), and studies on their inheritance *per se* (Bhojwani *et al.* 1977)

— restoring ploidy level in polyploid species after breeding at reduced ploidy level (Waara and Glimelius 1995)

— direct production of allopolyploids (amphidiploids), so-called "resynthesis" in cruciferous plants, without chromosome doubling (Campbell 1993)

— transfer of partial nuclear information from one species to another (Bajaj 1994)

— production of the heteroplasmic state in the extra-nuclear genetic elements in addition to nuclear hybridity (Kameya *et al.* 1989, Navrátilová 2004)

— induction of somaclonal variants (Bajaj 1994)

However, somatic hybridization is a random genomic recombination process and the genome composition of the somatic hybrids is not well-known (Wang *et al.* 2008). Moreover, even if barriers preventing sexual hybridization between two species are bypassed by protoplast fusion, barriers may still exist at the somatic level. Consequently, the differentiation, growth and development of different vital organs, such as roots and flowers as well as gamete production may be blocked, thereby inhibiting production of hybrid plants (Fahleson *et al.* 1994). Therefore, it may limit the possibilities to combine distantly related species into functional hybrids (Forsberg *et al.* 1994).

In most cases, fusion of two divergent parents leads to hybrids that combine nuclear genomes from both fusion parents, resulting in regeneration of symmetric hybrids. Incorporation of total genomes of the two parents, especially nuclear ones, in a hybrid has two obvious disadvantages, introduction of too much exotic genetic material accompanying the expected gene(s) and genetic imbalance leading to somatic incompatibility (Kumar and Cocking 1987, Liu *et al.* 2005, Tu *et al.* 2008). Challenges with the heredity of above mentioned somatic hybrids between distant species, such as incompatibility of genome recombination, poor viability and infertility are the most common (Kirti *et al.* 1991, Waara and Glimelius 1995, Hansen and Earle 1997, Brewer *et al.* 1999, Wang *et al.* 2003, Liu *et al.* 2007). Thus, in order to transfer only a limited part of a genome of one species to the genome of another species, the technique of asymmetric somatic hybridization has been developed (Dudits *et al.* 1980; see Chapter Protoplast fusion).

Although the somatic hybridization technique overcomes part of problems connected with sexual hybridization and offers additional benefits, its usefulness relies on the ability to regenerate plantlets that can be grown to maturity (Hansen and Earle 1994a, Vicient and Martínez 1998). Therefore, efficient plant regeneration system from fused protoplasts is first required (Cai *et al.* 1996, Hansen *et al.* 1999, Hu *et al.* 1999, Davey *et al.* 2005). Despite extensive research over the past decades, regeneration from protoplasts has been observed in only a limited number of plant species. Protoplasts from important agricultural crops often show recalcitrance to the regeneration of a whole plant (Watanabe *et al.* 2002, Yasuda *et al.* 2007). Concerning cruciferous crops, although plant regeneration has largely been illustrated in various species of several genera, studies are always required to improve this technique to make it applicable to new genotypes (Delpierre and Boccon-Gibod 1992).

It can be concluded, that optimization of all crucial steps (i.e., protoplast isolation and fusion, identification of fusants, protoplast culture, whole plant regeneration and selection of plants with a hybrid progeny) of the somatic hybridization technique is an absolute prerequisite for its successful and routine implementation into breeding programmes of agricultural crops.

2. Protoplast Isolation

2.1. History

Since Klercker in 1892 (Bhojwani and Razdan 1996) first isolated protoplasts mechanically from water pineapple (*Stratiotes aloides*), numerous attempts have been made to isolate vital protoplasts from plant tissues. Considerable progress in term of the yield and viability of obtained protoplasts was accomplished via the enzymatic digestion of the plant tissue. Cocking (1960) used cellulase from the fungus *Myrothecium verrucaria* for releasing protoplasts from root tips of tomato seedlings. This method was later improved by Takebe *et al.* (1968), who introduced two-step enzymatic procedure to isolate mesophyll protoplast of tobacco. The leaf pieces were first exposed to macerozyme to release single cells, which were then treated with cellulase to digest the cell walls and release the protoplasts. Power and Cocking (1970) simplified above mentioned method by using the mixture of commercially available pectinases and cellulases to isolate protoplasts from tobacco leaves. In conclusion, mechanical procedures, involving slicing of plasmolysed tissues, are now rarely employed for protoplast isolation, but are useful with large cells and when limited (small) numbers of protoplasts are required (Davey *et al.* 2005).

2.2. Source of protoplasts

Protoplasts can be isolated from a variety of plant tissues and organs, including hypocotyls, leaves, stems, shoot apices, roots, fruits, coleoptiles, aleurone layer of cereal grains, root nodules, microspore mother cells, microspore tetrads, and pollen tubes (Ahuja 1982).

In the genus *Brassica*, following tissues have been used the most frequently:

— hypocotyls (Barsby and Shepard 1983, Barsby *et al.* 1986, Ohlsson and Eriksson 1988, Kameya *et al.* 1989, Thomzik and Hain 1990, Hegazi and Matsubara 1992, Forsberg *et al.* 1994, Brewer *et al.* 1999, Hu *et al.* 1999, Chen *et al.* 2001, 2004, Ishikawa *et al.* 2003, Scholze *et al.* 2010)

— leaves (Kameya and Takahashi 1972, Kao and Seguin-Swartz 1987, Loudon *et al.* 1989, Jourdan *et al.* 1990, Sigareva and Earle 1999, Hansen *et al.* 1999, Hu *et al.* 1999, Chen and Halkier 2000, Ren *et al.* 2000, Chen *et al.* 2004, Wang *et al.* 2005, Du *et al.* 2009, Scholze *et al.* 2010)

— cotyledons (Barsby and Shepard 1983, Ohlsson and Eriksson 1988, Robertson *at al.* 1988, Kameya *et al.* 1989, Jaiswal *et al.* 1990, Hegazi and Matsubara 1992, Ishikawa *et al.* 2003, Chen *et al.* 2004, 2005)

On the contrary, petioles (Chen *et al.* 1994), pollen tetrads (Wakasa 1973), microspores (Sun *et al.* 1999, Liu *et al.* 2007), mature pollens (Minami *et al.* 1995), suspension cultures (Wang *et al.* 2008, Prange *et al.* 2010), calli (Wakasa 1973), roots (Xu *et al.* 1982) and root tips (Kameya and Takahashi 1972), stem embryos (Kohlenbach *et al.* 1982), stems (Chuong *et al.* 1987a) and stem cortex (Klimaszewska and Keller 1987) or an inflorescence (in cauliflower) (Yang *et al.* 1994) have been used as a minor source for the protoplast isolation.

Concerning *in vivo* grown sources, stem sections can be subjected to rigorous surface sterilization procedures, contamination of the protoplast cultures is not a problem if the original plant is disease-free. Additionally, the amount of stem material that can be obtained from one plant can be increased by trimming the old raceme to induce new ones to develop (Chuong *et al.* 1987a). Wakasa (1973) mentioned difficulties with the isolation of protoplasts from mature pollen grains, because their exines could not be digested by any enzymes used. Although there have been attempts to isolate protoplast from younger pollen grains (microspores), the yield is still not sufficient enough for routine use (Sun *et al.* 1999, Liu *et al.* 2007). Seasonal variation in physical factors, which affects the reproducibility of protoplast isolation from *in vivo* grown plants, is generally eliminated using *in vitro* grown material (Davey *et al.* 2005).

Thus, the most suitable material for protoplast isolation is seedling tissue (hypocotyls, green cotyledons, or leaves) grown *in vitro* or in growth chambers (Jourdan 1994, Hu *et al.* 1999). Seedlings have the advantage that protoplasts can be isolated from their tissues within a few days of seed germination (Davey *et al.* 2005). The main disadvantage of young seedlings, however, include limited yields, production of highly heterogeneous protoplast populations, and the requirement for large numbers of seedlings thereby increasing the probability of microbial contamination (Klimaszewska and Keller 1987). Therefore, the preferred tissue source for protoplast isolation are fully developed true leaves (Bidney *et al.* 1983), since the plants can be easily grown in long-term aseptic cultures, leaves can be generated in large amounts and maintain high uniformity of isolated protoplasts (Bhojwani *et al.* 1977, Loudon *et al.* 1989), even though the mesophyll protoplasts of *Brassica* species (contrary to hypocotyl protoplasts) have been found more recalcitrant to culture (Ulrich *et al.* 1980). Moreover, although leaf tissues give high yields of protoplasts, the viability is dependent on the physiological condition of the donor plants and it is therefore difficult to consistently obtain viable protoplasts at high frequencies (Klimaszewska and Keller 1987).

On the other hand, isolating protoplasts from suspension culture has certain advantages as these cells are grown under carefully controlled nutritional and physical conditions, and the requirements for their growth and morphogenesis are already known (Ahuja 1982). However, suspension and/or *in vitro* long-term cultures are involved by soma- and protoclonal variation, restrained the uniformity of isolated protoplasts (Kawata and Oono 1998).

In conclusion, hypocotyls of young seedlings and leaves from young, in a greenhouse grown plants or shoots grown *in vitro* are the most commonly used source of protoplasts, providing a reasonably good source of protoplast material in term of the protoplast yield and viability (Ahuja 1982).

2.3. Isolation of protoplasts

2.3.1. Release of protoplasts

Two common methods have been used for releasing protoplast from the tissue:

(i) The mechanical methods basically involve strip-cutting of plasmolysed plant tissues followed by induced osmotic swelling to release the protoplasts. The number of protoplasts obtained by such methods is rather limited, and the procedure can be adapted for only a few types of tissues, including highly vacuolated cells (e.g. onion bulbs, scales, radish roots), which may not be necessarily suitable for continued growth in culture. The mechanical method is used only occasionally, but has a merit in that the unknown effects of macerating and cell wall degradation enzymes on protoplast and its contents are eliminated (Ahuja 1982).

(ii) Chemical methods, i.e. resolving the cell wall components of the plant tissue by various fungal enzymes to liberate protoplasts, are now used predominantly. Due to chemical digestion, its improvement and the use of commercially available enzymes is now possible to isolate protoplasts from virtually every organ and tissue of the plant, provided of course that the walls have not undergone extensive secondary thickening (Bhojwani *et al.* 1977).

Various enzymes (cellulases, hemicellulases and pectinases, isolated from fungi *Trichoderma viride*, *Irpex lacteus*, *Aspergillus niger* and *Rhizopus* ssp.) and their mixtures have been used to obtain viable protoplasts. In most instances the crude commercial preparations of enzyme solutions have been used without any further purification. Very highly purified or crystalline enzyme preparations are less suitable for protoplast isolation, as these are unable to break down the chemically and structurally complex plant cell wall (Ahuja 1982). Usually the one-step procedure [introduced by Power and Cocking (1970)] comprising the combination of a pectinase, digesting the pectin-rich middle lamella, and a cellulase, melting primary and secondary cell walls, has been used in most of the experiments. For example, Chen *et al.* (1994) used the mixture of 1.5% cellulase Onozuka R-10 and 0.3% pectinase Macerozyme R-10 for the digestion of *B. napus* petiolar tissue; Chen *et al.* (2004) used 0.5% cellulase Onozuka RS and 0.1% pectinase Pectolyase Y23 to isolate protoplasts from hypocotyls of the red cabbage (*B. oleracea* var. *botrytis*) and Glimelius (1984) 1% cellulase Cellulysin and 0.1% pectinase Macerase in several genotypes of *B. napus*, *B. oleracea* and *B. rapa*; Jaiswal *et al.* (1990) macerated cotyledons of *B. carinata* by means of 0.3% cellulase

Meicelase and 0.02% pectinase Pectolyase Y23. Scholze *et al.* (2010) used the combination of 2% cellulase, 0.5% driselase, 0.5% hemicellulase and 1% pectinase for the liberation of protoplasts from the mesophyll and 2% cellulase and 0.3% macerozyme from the hypocotyl tissue in various genotypes of *B. oleracea*. Additionally, Jourdan (1994) recommended the mixture of three enzymes (Cellulysin, Macerase and Driselase) for consistent release of protoplasts from leaves and hypocotyls of *B. rapa* and *B. oleracea*.

To facilitate the penetration of enzyme solution into the intercellular spaces of the tissue, which is essential for successful digestion, various procedures have been used. A most commonly used one is to peel the lower epidermis and float the cutted pieces of leaf on the enzyme solution in a manner that the peeled surface is in contact with the solution (Bajaj 1974, Bhojwani and Razdan 1996) or to cut leaves and hypocotyls with sharp razor into small, 2–3mm segments (Glimelius 1984, Barsby *et al.* 1986, Kameya *et al.* 1989, Jaiswal *et al.* 1990, Chen *et al.* 1994, Chen and Halkier 2000, Scholze *et al.* 2010). To ease the liberation of protoplast from the tissue, gentle agitating on a shaker is often used during or after the enzymatic digestion. Namely, Mandal and Sikdar (2003) shaked the enzymatic mixture with the tissue for 11 hours at 50 rpm, Wang *et al.* (2008) for 2 hours at 110 rpm during incubation, Brewer *et al.* (1999) shaked the protoplasts continuously at 40 rpm during long-term (18 h) enzyme treatment and Scholze *et al.* (2010) overnight at 30 rpm.

Fundamental property of isolated protoplasts is their osmotic fragility and, hence, the need for a suitable osmotic stabilizer in the enzyme solution (Bhojwani and Razdan 1996, Navrátilová 2004). To increase the stability of protoplasts, inorganic salts (Ca^{2+}) and organic buffer (e.g. morpholinoethane sulphonic acid) are added along with non-ionic osmotica to minimize the changes of pH during incubation (Navrátilová 2004).

The most frequently used non-ionic osmotica are mannitol (Larkin 1976, Shillito *et al.* 1983, Chatterjee *et al.* 1985, Köhler *et al.* 1989, Hu *et al.* 1999, 2002, Mandal and Sikdar 2003, Chen *et al.* 1998, 2004, Chakraborty and Sikdar 2008, Jiang *et al.* 2010, Scholze *et al.* 2010), sorbitol (Shillito *et al.* 1983, Robertson and Earle 1986, Hansen and Earle 1994b, Schirawski *et al.* 2000, Scholze *et al.* 2010), sucrose (Chen *et al.* 1994, Papadakis *et al.* 2005) and glucose (Narasimhulu *et al.* 1992, 1993) or their combinations (Minami *et al.* 1995). Ionic osmotica (various inorganic salts) are less preferable; for example Ishikawa (2010) obtained good results with the mixture of 0.6 mol l^{-1} NH_4Cl and 0.37 mol l^{-1} NaCl. Hsiao and Bornman (1989) observed slight morphological differences among protoplasts under different osmotica. Mesophyll protoplasts in the presence of mannitol had their chloroplasts more or less evenly distributed throughout the cytoplasm. In contrast, in the presence of sucrose, chloroplasts were clustered around the nucleus.

2.3.2. Purification of protoplast suspension

The mixture of intact and viable protoplasts after maceration usually contains various other fragments, namely chloroplasts, vascular elements, undigested cells and damaged protoplasts. It is therefore necessary to remove these unwanted particles. Thus, released protoplasts are typically filtered through nylon or metal meshes 50–150 µm in size and further purified by several cycles of low speed centrifugation and resuspension (Chatterjee *et al.* 1985, Jourdan 1994).

Considerable progress in term of purity, yield and viability of isolated protoplasts has been achieved via establishing of the gradient purification method, where protoplasts flotate on dense sucrose, Ficoll or Percoll gradients (Attree and Sheffield 1986, Loudon *et al.* 1989, Millam *et al.* 1991, Kirti *et al.* 1992a, Harter *et al.* 1993, Endler *et al.* 2006, Liu *et al.* 2007). The centrifugation speed vary from 50 to 200 *g*, two or three centrifugation cycles (5–10 minutes each) are usually sufficient to obtain purified suspension of viable protoplasts. For example, Taniguchi *et al.* (1994) filtered the suspension through nylon mesh and then centrifuged at 100 *g* for 5 minutes. After centrifugation, precipitated protoplasts were washed three times in washing solution in a centrifuge at 50 *g* for 2 min. Cai *et al.* (2010) purified the suspension through a 45µm stainless steel mesh, followed by centrifugation at 100 *g* for 3 min on a 25% sucrose/13% mannitol gradient. Liu *et al.* (2007) used 30% sucrose/W5 gradient for discontinuous centrifugation with a lower speed of 500 rpm (40 *g*) for 12 min. Scholze *et al.* (2010) filtered the suspension through a nylon mesh (40 µm) and centrifuged five minutes at 100 *g*. Protoplast pellets were then resuspended and centrifuged on the sucrose/ /mannitol-sorbitol gradient 10 min at 100 *g*. Finally, the floating band of protoplasts was washed twice with protoplast culture medium. Loudon *et al.* (1989) peleted the protoplasts by centrifugation at 200 *g* for 7 min, resuspended in fresh washing solution and again centrifuged for 5 min. Protoplast were then purified on a 30% Percoll gradient at 100 *g* for 5 min.

2.3.3. Yield and viability of protoplasts

High yields of viable protoplasts are necessary for somatic hybridization experiments (Kirti and Chopra 1990). Although the protoplasts can be isolated easily, their yield and viability depends on a number of factors, the most important ones being the type of the tissue, the age and the physiological state of the plant. In addition, particular attention should be paid to the concentration and purity of the enzyme, their pH, period of incubation, and specially the plasmolyticum as they immensely affect the viability of the protoplasts (Bajaj 1974).

For example, Zhao *et al.* (1994) found evident genotypic differences in yield from cotyledons of *B. rapa*, which appeared to be associated with the different conditions of cotyledon germination and the varieties used in protoplast isolation. The highest yield (in cv. Bunyip) was 8.3×10^4 protoplasts per one cotyledon. Jaiswal *et al.* (1990) tested the effect of seedling age on the isolation of cotyledon protoplasts of *B. carinata*. The highest yield of protoplasts (9.0×10^4 per cotyledon) was obtained from 5 day old seedlings, while only 4.08×10^4 and 1.00×10^4 in 11 or 21 day old seedlings, respectively. Hegazi and Matsubara (1992) also found significant effect of the seedling age and the genotype on the yield from cotyledons of radish (*Raphanus sativus*) and pointed out, that seedling age is critical factor in achieving high protoplast yield.

In hypocotyls, the results of Chen *et al.* (2004) showed that the hypocotyls of 3–5 day old seedlings offered enough good quality protoplasts for culture, that is, around 1.8×10^4 per seedling. Gupta *et al.* (1990) obtained the best yield from 6 day old hypocotyls of *B. nigra,* Narasimhulu *et al.* (1992) found the highest yield in 7 day old hypocotyls of *B. carinata* (around 1.0×10^4 per seedling), while in 5 d and 10 d old seedlings the yield was lower and the viability was considerably reduced in the latter one. On the contrary, 3 day old hypocotyls of *B. napus* gave the most reproducible yields (3×10^6 protoplasts per seedling) (Barsby *et al.* 1986).

In general, older seedlings had reduced yields, which may be due to secondary wall components resistant to enzyme digestion (Barsby and Shepard 1983) or viability, due to a higher frequency of vacuolated protoplasts in hypocotyls (Narashimulu *et al.* 1992). Fahleson and Glimelius (1999) found a variation on the yield of hypocotyl protoplasts also among different species and even varieties.

It is easier to obtain enough material from green leaves than from other organs (Fahleson and Glimelius 1999). For example, Hansen and Earle (1994b) isolated protoplasts from the young *in vitro* grown leaves and the yield ranged between 2 and 12×10^6 protoplasts per g of leaf tissue. Cohen *et al.* (1981) obtained the highest yield [3–5 $\times$ l0^6 per gram of the fresh weight (FW)] from rapidly expanding leaves of one month old plants of Chinese cabbage (*B. rapa* var. *pekinensis*) and Delpierre and Boccon-Gibod (1992) around 10 $\times$ l0^6 per gram of FW from leaves of cauliflower (*B. oleracea* var. *botrytis*).

Generally, the youngest fully expanded leaves from several week-old*, in vitro* grown plants or 3–7 day old seedlings (to obtain both hypocotyls and cotyledons) give the highest yield of protoplasts in respect to the type of the tissue (Wang *et al.* 2005, Hansen *et al.* 1999, Hu *et al.* 1999, Sigareva and Earle 1999, Ren *et al.* 2000, Chen *et al.* 2004, Du *et al.* 2009). Therefore, the yield per gram of FW vary between 2–50 $\times 10^6$ in case of true leaf tissue, 3–11 $\times 10^6$ in seedling cotyledons and only 0.2–2 $\times 10^6$ in case of hypocotyls or 1–2.5 $\times 10^6$ in calli (Cardi and Earle 1997, Hegazi and Matsubara 1992).

Although the yield of protoplasts is significantly affected by plant organs and seedling ages, these factors interacted with enzyme type, incubation period and osmoticum concentrations. For example, in the experiments of Hegazi and Matsubara (1992) with radish cotyledons, the highest protoplast yield was obtained from the combination of Cellulase Y-C and Pectolyase Y-23 in most cultivars. However, cultivar "Harumaki Minowase" gave the highest yield after a treatment with Cellulase Onozuka RS and Pectolyase Y-23.

Castelblanque *et al.* (2010) used 0.4% Cellulase Onozuka R-10 and 0.2% Driselase to isolate the large amount of viable protoplasts. Concerning the isolation of protoplasts from microspores of *B. napus*, 3h incubation with 1.0% cellulase, 0.8% pectinase, 0.02% pectolyase, and 0.3% macerozyme gave best results, considering both isolation frequency and protoplast viability (Sun *et al.* 1999). Narasimhulu *et al.* (1992) obtained the highest yields after 16 h incubation of *B. carinata* hypocotyl segments in the enzyme solution, consisted of 0.5% cellulase and 0.025% pectolyase. Although higher concentrations of these enzymes released protoplasts in a shorter incubation period (12 h), resulted in lower yields and viability, possibly due to toxic effects associated with increased concentration of enzymes. Brewer *et al.* (1999) in *B. napus* successfully liberated protoplast from leaf tissue in the enzymatic mixture (1% Cellulysin and 0.1% Macerase) after 16–18 h treatment, Hansen and Earle (1994b) in rapid cycling *B. oleracea* and Ren *et al.* (2000) in broccoli (*B. oleracea* var. *italica*) obtained high yields of mesophyll protoplast after long-term (18 h) enzyme digestion with 2% Cellulysin, 0.5% Driselase and 1% Macerozyme.

Narasimhulu *et al.* (1992) tested suitable osmotica in term of the yield and viability of protoplasts. Four different concentrations (0.3, 0.4, 0.5 and 0.7 M) of mannitol and glucose were tested separately. Protoplasts with higher viability and minimal budding were obtained at a concentration of 0.4 M osmoticum. The two osmotica tested did not show statistical differences for percentage viability or yield. Mannitol was chosen for the enzyme mixture because it should not provide an efficient energy source during digestion. In other experiments of Narasimhulu *et al.* (1993), mannitol was preferred in the enzyme mixture as well, as it resulted in higher yield compared to glucose, though the latter gave a higher viability (77.7%). Additionally, Hegazi and Matsubara (1992) identified 0.5 M mannitol as the best concentration, while Sun *et al.* (1999) observed, that the minimum concentration was 0.7 M, at which a considerable number of protoplasts remained intact.

The viability of protoplasts can be determined by several methods. The most reliable test is the regeneration of the cell wall and subsequent cell divisions. The presence of active cytoplasmic flowing in freshly isolated protoplasts can also be used as a sign of viability (Ahuja 1982). Most commonly used procedure, especially in connection with the protoplast fusion techniques is vital staining of isolated protoplasts,

namely with fluorescein diacetate (Craig *et al.* 1997, Liu *et al.* 2007), Evans blue (Nea and Bates 1987) or neutral red (Kameya 1975); early regeneration of the cell wall can be detected by means of Calcofluor White staining (Nedukha 1998).

Contingent fusion procedure must follow immediately after the removal of protoplasts from the enzyme solution for maximum fusion frequency, as cell wall regeneration occurs very rapidly (Ahuja 1982).

3. Protoplast Fusion

3.1. History

Fusion between isolated protoplasts of different species which is the basis of somatic hybridization requires a fusion inducing agent (Bhojwani *et al.* 1977). Although there were attempts to fuse protoplast via a mechanical method (bringing of protoplasts into contact by micromanipulation equipment), the frequency of fusion was insufficient (Schenk and Hildebrandt 1971). Later on, the fusogen on the basis of sodium nitrate (Power *et al.* 1970, Carlson *et al.* 1972, Kameya and Takahashi 1972), immune sera (Hartman *et al.* 1973), gelatin (Kameya 1973) and dextran sulfate (Kameya 1975) were used to agglutinate and fuse protoplasts; however the fusion frequency and the viability of fusion products were low. Finally, Keller and Melchers (1973) obtained high fusion frequencies and good viability with high concentrations of Ca^{2+} and basic pH.

Nevertheless, the most commonly used chemical method has been the procedure of Kao and Michayluk (1973), who first introduced nontoxic fusogen polyethylene glycol (PEG), combined with the presence of Ca^{2+} ions and basic pH. Another promising fusion technique has been established by Senda *et al.* (1979), who first used electric pulses to fuse plant protoplasts. The procedure of electrofusion has been further optimised by Zimmermann (1982) for routine use. Wiegand *et al.* (1987) introduced electroporation and protoplast fusion with the help of ultra-violet laser microbeam, applicable for the fusion of two preselected cells in a tissue. However, this method has so far failed to a greater extent.

3.2. Mechanism of fusion

Fusion of protoplasts consists of three main phases: (i), adhesion (agglutination), during which the plasmatic membrane of two or more protoplasts get in close contact after elimination of negative charges on the protoplast surface via fusogens, (ii), membrane fusion at small regions, establishing bridges between protoplasts and, (iii), forming a spherical fusant (a homo- or heterokaryon) due to expansions of cytoplasmic bridges.

3.3. Spontaneous fusion

Spontaneous fusion is an uncontrolled fusion of two or more protoplasts, that usually takes place during protoplast isolation. For instance, Ye and Earle (1991) observed spontaneous fusion in maize protoplasts, Hansen and Earle (1994a) in *B. oleracea*. Nea and Bates (1987) after using of 2% Cellulysin (without Driselase) did not speed

protoplast release, but reported extensive clumping of the protoplasts and possibly spontaneous fusion as well. To reduce this phenomenon, plasmolysis of the source tissue in salt or sugar solutions prior to enzymatic digestion is sometimes used (Larkin 1978, Gürel *et al.* 2002, Nugent *et al.* 2006, Prange *et al.* 2010). In conclusion, spontaneous fusion has no significance in somatic hybridization, which requires fusion between protoplasts of different origin (Bhojwani *et al.* 1977).

3.4. Induced fusion

3.4.1. Chemical fusion

Polyethylene glycol has been evaluated as a widely accepted chemical fusogen, because of the reproducible high frequency of binucleate heterokaryons (i.e. a fusant, consists of two genetically different nuclei) and low toxicity to most cell types. Concentration of a PEG solution in most of the experiments varied between 12–40%, and the molecular weight between 1500–8000. While the PEG treatment induces agglutination of the protoplasts, the fusion will occur after dilution of the PEG with a solution containing a high concentration of calcium ions at high pH (Bajaj 1994, Waara and Glimelius 1995, Scholze *et al.* 2010).

For instance, Kao *et al.* (1974) obtained high frequency of heterokaryons with high-molecular-weight PEG 1540 (MW) or 4000. However, the frequency of heterokaryons was also affected by the types of enzymes used for cell wall degradation, duration of enzyme incubation and molality of the PEG solutions. Kirti *et al.* (1992b) regenerated intergeneric somatic hybrids of *Trachystoma ballii* and B. *juncea* with using of 20% PEG (MW 8000) and 10% dimethyl sulfoxide (DMSO) and Kirti *et al.* (1995) somatic hybrids between *Diplotaxis catholica* and *B. juncea* with above mentioned PEG/ /DMSO solution. Narasimhulu *et al.* (1992) obtained resynthesized *B. carinata* after fusion by means of 20% polyethylene glycol 8000 and 10% DMSO. Menczel and Wolfe (1984) observed that DMSO significantly increased the frequency of PEG-induced fusion between *B. napus* and *B. carinata* protoplasts. Liu *et al.* (2007) reported 20% fusion frequency when used 40% PEG 4000 between pollen and haploid mesophyll protoplasts of *B. oleracea* and *B. rapa.* The lowest fusion frequency of about 4.0% was seen with 30% PEG 8000. Navrátilová *et al.* (1997) recovered intergeneric somatic hybrids between *B. oleracea* and *Armoracia rusticana* after fusion with PEG 6000. Robertson *et al.* (1987) obtained somatic hybrids from fusion between *B. oleracea* and atrazine-resistant *B. rapa* with 33% PEG 6000 and Yamagishi *et al.* (1992) with 30% PEG 4000. Scholze *et al.* (2010) obtained somatic hybrids from fusion between *B. oleracea* and various wild relatives when used 12.5% PEG 6000 for 5 min.

3.4.2. Electrofusion

The term electrofusion refers to the protoplast fusion that is induced by the application of electric current. Protoplasts are initially resuspended in the medium of low conductivity and pipetted between the two electrodes. A high frequency (0.5–1.5 MHz) alternate (AC) current (10–200 V cm^{-1}) is applied. The charge on the protoplast surface becomes polarized and thus protoplasts act as dipoles and move to a area of highest field intensity. As the protoplasts have been aligned in chain, one or two short (10-20 µs) direct (DC) pulses of high voltage (0.125–1 kV cm^{-1}) are applied which causes reversible membrane breakdown in the contact area of the adjacent protoplasts. The AC current is shortly applied again to provide close protoplast contact. The fusion process takes about 10 minutes (Bhojwani and Razdan 1996).

Brewer *et al.* (1999) aligned protoplasts of *Thlaspi caerulescens* and *B. napus* using AC current with a frequency of 1 MHz and a field strength of 0.125 kV cm^{-1} for 25 s. Cells were then fused by one or more 100µs DC pulses. Two electrofusion DC pulses at 1.25 kV cm^{-1} produced the highest heterofusion rate of 13% and good viability of fused protoplasts. Craig and Millam (1995) performed electrofusion of *Lunaria anua* and *B. napus* protoplasts under conditions of an AC current of 12 V, with a 110 V pulse of 50µs duration. Wang *et al.* (2005) used electrofusion to prepare somatic hybrids of *B. napus* and *Sinapis alba* with the following parameters: AC current 1.8 MHz; DC field, 150–160 V and 15 µs duration.

3.5. Symmetric and asymmetric fusion

3.5.1. Definition

The terms symmetric and asymmetric fusants refer to the genomic constitution of fusion products: symmetric protoplast fusion produces cells that contain a mixture of the DNA-containing organelles (nuclei, chloroplasts, and mitochondria) from both fusion parents, while asymmetric one results from spontaneous or induced rearrangements and/or a loss of DNA belonging to one of the fusion partners, regardless of whether they occur before or after the fusion process.

3.5.2. Symmetric fusion

Successful symmetric fusion between distant species were performed for example by Pelletier *et al.* (1983) between *B. rapa* and *R. sativus; B. juncea* × *Moricandia arvensis* (Kirti *et al.* 1992), *B. carinata* × *Camelina sativa* (Narasimhulu *et al.* 1994), *B. oleracea* × *Armoracia rusticana* (Navrátilová *et al.* 1997), *B. nigra* × *Raphanus*

sativus (Arumugam *et al.* 2002) and *B. napus* × *Orychophragmus violaceus* (Vasilenko *et al.* 2003). However, the formation of a symmetric fusant does not necessarily lead in the regeneration of a symmetric hybrid (see Chapter Spontaneous asymmetrization) since full genome complements are not present in a number of somatic hybrids derived from symmetric fusion (Liu *et al.* 2005).

3.5.3. Resynthesis of allotetraploid species

Investigations of U in 1935 (Campbell 1993) elucidated the genetic relationships among the six economically most important *Brassica* species (Figure 1). The cultivated *Brassica* species involve three diploid species, *B. rapa* (A genome, 2n = 20), *B. oleracea* (C genome, 2n = = 18) and *B. nigra* (B genome, 2n = 16), and three allotetraploid (amphidiploid) species, *B. napus* (AC genomes, 2n = 38), *B. juncea* (AB genomes, 2n = 36) and *B. carinata* (BC genomes, 2n = 34). U in 1935 (Poulsen *et al.* 1993) illustrated the amphidiploid nature of *B. juncea, B. carinata* and *B. napus* and showed that these genotypes could be constructed (resynthesised) by gametic crossing of their diploid predecessors. Allotetraploid species have been created also with the help of protoplast fusion, resulting in symmetric somatic hybrids: *B. napus* (Schenck and Röbbelen 1982, Sundberg and Glimelius 1986, Taguchi and Kameya 1986, Terada *et al.* 1987, Robertson *et al.* 1987, Chen *et al.* 1988, Rosén *et al.* 1988, Jourdan *et al.* 1989, Heath and Earle 1995), *B. carinata* (Narasimhulu *et al.* 1992) and *B. juncea* (Yadav *et al.* 2009).

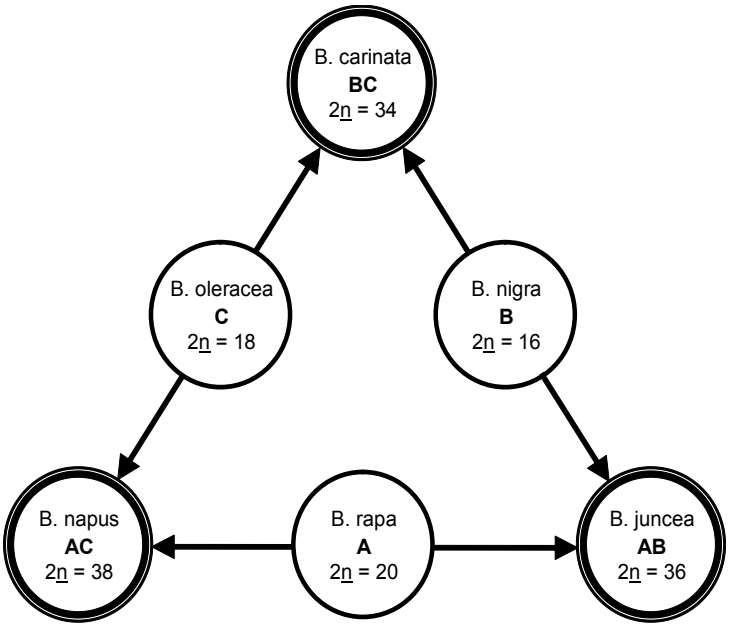

Figure 1: Triangle of U demonstrating genome relationships among agriculturally important *Brassica* species [Adapted from U (1935)]

3.5.4. Spontaneous asymmetrization

After the symmetric fusion between parents of unrelated species, parts of genetic information of one or both genomes are often being lost during the *in vitro* cultivation procedure, resulting in asymmetric hybrids (Negrutiu *et al.* 1989). However, the factors, affecting the spontaneous induction of such asymmetrization are not known and therefore unpredictable (Hinnisdaels *et al.* 1994).

3.5.5. Induced asymmetrization

The disadvantages of symmetric hybrids between distant species (see Introduction) have been partially solved by asymmetric hybridization. However, viable and fully functional hybrid plants between unrelated species require an extensive asymmetrization (Hinnisdaels *et al.* 1994). Thus, a more realistic approach than the combination of complete, highly diverse genomes between distantly related species is the transfer of small units of genetic material from donor to recipient protoplasts (Hoffmann and Adachi 1981).

The most widely used method is the irreversible fragmentation of nuclear chromosomes via X- or γ-irradiation of the donor protoplasts before fusion with normal protoplasts of the recipient parent. The possible application of UV-irradiation provides an attractive alternative to ionizing-type irradiations as well (Hinnisdaels *et al.* 1994). Another method to eliminate nuclear DNA of donor protoplasts is their enucleation via ultracentrifugation in a percoll/mannitol gradient (Sigareva and Earle 1997). For instance, Gerdemann-Knörck *et al.* (1995) reported asymmetric fusion between *B. napus* and *B. nigra* protoplasts, where the latter were irradiated with X-ray doses ranging from 450 to 1300 Gy. Scholze *et al.* (2010) obtained asymmetric hybrids when irradiated donor mesophyll protoplasts with X-ray dose of 92 Gy before fusion.

Forsberg *et al.* (1998a) evaluated several doses of UV-irradiation to produce asymmetric hybrids. Protoplasts of *A. thaliana* were irradiated with doses between 780 and 28 080 J m^{-2} and fused with protoplasts of *B. napus*. Plants with a hybrid progeny were obtained using doses of 780, 2340 and 4680 J m^{-2}. Forsberg *et al.* (1998b) compared the efficiency of UV- and X-irradiation on the frequency of asymmetric fusion between *A. thaliana* and *B. napus*. Both UV- and X-irradiation proved to be powerful tools in the production of asymmetric somatic hybrids. The frequency of asymmetric hybrid plants produced varied between 57% and 100% in the experiments.

Dushenkov *et al.* (2002) obtained asymmetric hybrids from fusion between *B. juncea* and *Thlaspi caerulescens* mesophyll protoplasts. The latter were irradiated before fusion by using an X-ray irradiation with the dose of 60 Krad. Sigareva and Earle (1997) tested the effectiveness of γ-, UV-irradiation and enucleation treatment to eliminate nuclear DNA in *B. oleracea* protoplasts. γ-irradiation was the most efficient,

while other treatments did not ensure good results. Moreover, the suspension after ultracentrifugation at 40 000 *g* still contained both cytoplasts (enucleated protoplasts) and complete protoplast and thus required further γ-irradiation treatment.

3.5.6. Cybridization

A type of asymmetric fusion, where fusion products ("cybrids") comprise of the nucleus of one parent, and extra-nuclear genome(s) (plastom, chondriom) of the other one or both parents. The process to obtain cells or plants with such genetic combination(s) is called cybridization. To ensure above mentioned hybrid constitution and to prevent cell division of unfused protoplasts of the recipient parent, the application of irreversible metabolic inhibitors (e.g. iodoacetate, iodoacetic acid) is always applied to the protoplast suspension to make them physiologically impaired for cell division when they are cultured independently. But when the protoplasts are fused with the donor protoplasts, the fusion products can grow due to metabolic complementation (Liu *et al.* 2005). Donor protoplasts are usually treated with ionizing or non-ionizing irradiation (see Chapter Induced Asymmetrization). Cardi and Earle (1997) obtained cybrids between *B. rapa* and *B. oleracea* and Hansen and Earle (1997) between *Sinapis alba* and *B. oleracea*, when irradiated the protoplast of the former ones during enzyme digestion with 30 krad γ-rays from a [^{137}Cs] source. Recipient protoplasts of the latter one were treated for 20–30 min at 30°C with 3–5 mM iodoacetate (IOA).

Hu *et al.* (2002) recovered cybrids from the asymmetric fusion experiments, where *B. napus* protoplasts were pre-treated with 3 mM IOA for 30 min and those of *Orychophragmus violaceus* were subjected to X-irradiation at doses of 100 and 200 Gy. Du *et al.* (2009) reported intertribal asymmetric fusion between *B. napus* and *Isatis indigotica*. *B. napus* protoplasts were treated by 3 mM IOA for 10 or 15 min to prevent the division of unfused protoplasts. Protoplasts of *I. indigotica* were subjected to UV irradiation for 5, 8, 10, 12, 15, 20 s to fragment its nuclear genome.

3.6. Identification of fusion products

3.6.1. Terminology

When the symmetric protoplast fusion takes place, two basic types of fusants can be obtained: (i), a homocaryon, which consists of two or more genetically congruous nuclei from only one fusion parent or (ii), a heterocaryon, consists of two or more nuclei, where at least one is genetically different from the other(s).

Similarly, a homo- or heteroplasmon involved the combination of genetically identical or different cytoplasmic organelles (mitochondria, plastids) of one or both fusion

parents after the constitution of symmetric or asymmetric hybrids and cybrids.
The terms “a homofusant” and “a heterofusant” simply refer to fusion products, where the former comprises genetically identical fused components, the latter genetically different fusion parents, regardless of the genomic and organellar constitution of fusion partners (e.g. protoplasts, cytoplasts, nucleoplasts etc.).

The terms “a multifusant” or “a polyfusant” refer to fused protoplasts, comprised of more than two fused cells. Only heterofusants consisting of two fused cells have a potential to ensure regeneration of “functional” somatic hybrids. Therefore, for plant breeding purposes, a selection of the fusion products is necessary (Glimelius 1988). Although hybrid plants were obtained without a selection method, this required the culture of large numbers of non-hybrid calli and regenerated plants (Brewer *et al.* 1999). Thus, to decrease the amount of *in vitro* cultivated fusion products, an efficient and reliable identification and/or selection method of heterofusants is clearly needed.

3.6.2. Identification and selection of heterofusants

Fairly convenient approach to identify heterofusants is to use visually different fusion parents. For example, fusion between green mesophyll and colourless hypocotyl (callus, cell suspension and root, respectively) protoplasts ensured early assessment of hetero- and homofusants and with the help of a micromanipulator the selection and the separate cultivation of desired fusants. Kameya and Takahashi (1972) reported easy distinction between leaf protoplasts and root protoplasts, because the former had green chloroplasts and anthocyan pigment. Additionally, protoplast can be labelled prior to fusion with nontoxic dye(s) and heterokaryons manually isolated via micromanipulator (Yarrow *et al.* 1986). Utilization of transgenic plants with gene(s) for fluorescence proteins (green, cyan, blue, yellow etc.) is another method to distinguish between homo- and heterofusants, and unfused protoplasts (Olivares-Fuster *et al.* 2002).

Brewer *et al.* (1999) discovered a possible selection method, based on the apparent differential adhesive capabilities of *B. napus* and hybrid cell colonies grown in liquid medium. Among fusion cell colonies, it was observed that some adhered to the bottom of the Petri dish (as did *B. napus* colonies), while others did not. DNA analysis of plants from the two populations indicated that a high proportion of plants derived from “floating” colonies were hybrids.

Microfusion (Glimelius 1988, Spangenberg 1994) facilitates the production of fusants with desired (hybrid) progeny since just a pair of protoplasts is fused by means of micromanipulation equipment. Therefore, no further selection is necessary. Concerning mass fusion, several techniques have been developed for selecting hybrid cell lines at early stages, including antibiotic resistance, temperature sensitivity, chlorophyll deficiency, metabolic inhibition of parental protoplasts with chemicals such as iodoac-

etate and fluorescence-activated cell sorting (Glimelius 1988, Morikawa and Yamada 1992). Glimelius (1988) routinely sorted out heterofusants produced between hypocotyl protoplasts from one species stained with carboxyfluorescein diacetate and mesophyll protoplasts, containing the fluorophore chlorophyll, from the parental cells.

Hu *et al.* (2002) employed the combination of irreversible chemical inhibition and *in vitro* recalcitrance, where one fusion parent was inactivated by IOA and the other had no capacity to regenerate in the protoplast culture. Thus, only heterofusants were then capable of further regeneration. The combination of chemical inhibition and ionic or non-ionic irradiation, where only fusion products with a hybrid constitution are able to regenerate in the protoplast culture represents another favourable selection method (see Chapter Cybridization).

4. Protoplast Culture

4.1. History

The first successful regeneration of fertile plants from protoplast cultures was performed by Takebe *et al.* (1971) in tobacco; Kartha *et al.* (1974) regenerated plants from protoplasts in rapeseed (*B. napus*) and first somatic hybrids were produced through symmetric fusion by Carlson *et al.* (1972) in tobacco. Since the middle 1970's, considerable progress has been made in the development of *Brassica* protoplast culture techniques (Zhao *et al.* 1994). For example, Gleba *et al.* (1978) reported successful protoplast fusion between *A. thaliana* and *B. rapa.* Although the "Arabidobrassica" regenerants, obtained in 1978 were the first flowering intertribal somatic hybrids in cruciferous plants, all regenerated plants remained sterile (Gleba and Hoffmann 1980). Preparation of cybrids between *B. napus* and *Raphanus sativus* published Pelletier *et al.* (1983) and the first resynthesis of *B. napus* Schenck and Röbbelen (1982).

Protoplast culture technology in *Brassica* species is being developed by an increasing number of investigators for a variety of purposes, including mutant isolation, somatic hybridization and genetic transformation. However, reliable protocols for protoplast culture and plant regeneration must be available in order to achieve such objectives (Kao and Seguin-Swartz 1987, Hansen *et al.* 1999).

4.2. Protoplast cultivation

4.2.1. Culture density

In addition to three major factors that influence the development of protoplasts in culture (the genotype, type of source tissue and its physiological state), the density of protoplasts in the culture medium is crucial for sustained mitotic division and further regeneration (Davey *et al.* 2005). Protoplasts are usually cultured at mean densities of $1 \times 10^4 - 1 \times 10^6$ protoplasts ml^{-1}. For example, Yarrow *et al.* (1990) used the fusion product density 1×10^5 ml^{-1} in somatic hybridization experiments between *B. napus* and *B. oleracea*, Zhao *et al.* (1994, 1995) above mentioned density in *B. rapa, B. napus* and *B. oleracea* protoplast cultures, Chuong *et al.* (1987b) 2×10^5 ml^{-1} in *B. carinata* and Ford (1990) in *A. thaliana,* Chatterjee *et al.* (1985) 4×10^4 ml^{-1} in *B. juncea,* Gupta *et al.* (1990) $2–5 \times 10^4$ ml^{-1} in *B. nigra* and Hegazi and Matsubara (1992) $2–7 \times 10^6$ ml^{-1} in *Raphanus sativus.*

The density of protoplasts can be decreased, if a feeder-layer (Walters and Earle 1990, Hu *et al.* 1999, Ren *et al.* 2000) or a nurse culture (Chuong *et al.* 1988, Simmonds *et al.* 1991, Chen *et al.* 2004) is used for the regeneration of recalcitrant species.

4.2.2. Cultivation media

Protoplast cultivation media contain major and minor elements, vitamins, myo-inositol, Fe-EDTA, sugar and growth regulators. Thus, the nutrient requirements of isolated protoplasts are very similar to those of cultured cells and tissues. However, in the absence of cell wall, protoplasts tend to be very efficient in the uptake of nutrients from the medium. For this reason, the initial nutrient media used for culture of protoplast must be modified to contain reduced levels of inorganic substances (Ahuja 1982); to prevent bursting of protoplasts, an extra amount of osmoticum is added (Bajaj 1974). Protoplasts can be grown and induced to divide both in liquid as well as in the agar or agarose solidified media (Bajaj 1974).

Additionally, protoplasts from different species and from different tissues of the same species generally vary in their nutritional requirements. Consequently, the medium most suitable for culture must be determined empirically (Davey *et al.* 2005). Several types of media and their modifications have been frequently used in cruciferous crops at initial stages of protoplast culture: KM 8p medium (Kao and Michayluk 1975), B and C (Pelletier *et al.* 1983), A (Glimelius *et al.* 1986), modified PS or PG (Ford 1990), K3 (Zhao *et al.* 1994), modified NN (Zhao *et al.* 1995) or modified MS medium (Chen *et al.* 2005). To initiate cell divisions and proliferation of the calli, cultivation media are supplemented with appropriate amounts of growth regulators at corresponding ratios (auxins *vs.* cytokinins). To stimulate rapid formation of dedifferentiated tissue, 2,4-dichlorophenoxyacetic acid (2,4-D) is the preferable auxin in early stages. For example, Cai *et al.* (1996) used modified liquid MS medium, containing 1.0 mg l^{-1} 2,4-D, 1.0 mg l^{-1} of 1-naphthaleneacetic acid (NAA) and 0.5 mg l^{-1} 6-benzyladenine (BA) for *Raphanus sativus,* Brewer *et al.* (1999) liquid KM 8p medium consisted of 1.0 mg l^{-1} 2,4-D, 0.1 mg l^{-1} of NAA, and 0.5 mg l^{-1} of 6-benzylamino purine (BAP) for *B. napus*, Zhao *et al.* (1994) liquid B medium with 0.5 mg l^{-1} 2,4-D, 0.1 mg l^{-1} NAA and 1 mg l^{-1} BAP for *B. rapa.*

Protoplasts in nutrient media are usually cultivated in the dark at 25°C to regenerate cell walls, initiate first divisions, microcolonies and small calli (microcalli). 2,4-D is replaced during callogenesis with less effective auxin [NAA, or indole-3-acetic acid (IAA)]. Viable calli are transferred onto regeneration media to initialize shoot regeneration and proliferation.

4.3. Whole plant regeneration

The ability to regenerate shoots from protoplast culture is foremost under genetic control (Jourdan *et al.* 1990, Chen *et al.* 2004, Yasuda *et al.* 2007). Despite extensive research during the past three decades, the regeneration from protoplasts has been observed in only a limited number of the many plant species studied to date. Protoplasts

of many important agricultural crops show recalcitrance in regeneration into whole plants (Yasuda *et al.* 2007).

Liquid or solid regeneration media, unlike those for callogenesis, usually contain lower amount of auxins and higher amount of cytokinins to stimulate regeneration of shoot primordia. Cytokinins as sole growth regulator sources could also stimulate shoot regeneration (Bonfils *et al.* 1992). Various media are used to meet these requirements, mainly modified MS medium (Barsby *et al.* 1986, Bonfils *et al.* 1992, Zhao *et al.* 1994, Sakai and Imamura 1994, Toriyama *et al.* 1994, Burbulis *et al.* 2008), modified K3 (Nagy and Maliga 1976), B5 (Delpierre and Boccon-Gibod 1992) or E and F media (Pelletier *et al.* 1983). For instance, Cai *et al.* (1996) obtained the best results (19% of calli formed shoots) on the MS medium, supplemented with 1.0 mg l^{-1} BA and 2.0 mg l^{-1} Kinetin in *Raphanus sativus*. Tian and Meng (1999) obtained the highest regeneration on the modified MS medium with 2.0 mg l^{-1} BA and 0.1 mg l^{-1} IAA in *Moricandia nitens*. Delpierre and Boccon-Gibod (1992) regenerated shoots from calli of *B. oleracea* var. *botrytis* on the modified B5 medium (2 mg l^{-1} BAP and 0.02 mg l^{-1} NAA). Hansen *et al.* (1999) reported shoot proliferation on solid E medium, supplemented with 1.0 mg l^{-1} of 2-isopentenyl adenine (2iP), 1.0 mg l^{-1} NAA and 0.02 mg l^{-1} of giberelic acid (GA_3) and Chen *et al.* (2004) regenerated plantlets of cabbage (*B. oleracea*) using modified MS medium with 1.0 mg l^{-1} BA and 0.2 mg l^{-1} NAA.

Shoots with developed leaves are transferred onto a rooting medium. To initialize root formation, agar solidified MS medium (Murashige and Skoog 1962) without growth regulators is predominantly used. In recalcitrant species the rooting can be stimulated by low concentration of an auxin in the MS medium [mainly indole-3-butyric acid (IBA)]. For example, Zhao *et al.* (1994) stimulated rooting via addition of 0.1 mg l^{-1} IBA, Brewer *et al.* (1999) and Du *et al.* (2009) with 0.1 mg l^{-1} NAA. Regenerants with properly developed roots are transferred to the peat substrate and covered with perforated foil to avoid over-drying of plantlets. Rapid transfer often gives more efficient recovery of vigorous plants (Earle 1994). The hybridity of plants can by assessed by means of various methods.

4.4. Identification of somatic hybrids

A critical component in successful somatic hybridization is an effective selection system, capable of allowing the recovery of a few true somatic hybrid colonies from a mixed population of regenerating protoplasts (Campbell 1993). Although possible somatic hybrids could be identified and separated immediately after fusion (see Chapter Identification of fusion products), an elimination of genetic material from the initial fusion product may take place and somaclonal variation might occur during the protoplast culture and/or whole plant regeneration (Glimelius 1988). Thus, a method

is required by which genetic material from both parents can be detected in order to prove its hybrid nature.

Initially, studies on morphology and chromosome numbers have been utilized to select desired products. However, these methods could be unreliable, since the morphology of plants may vary due to the polygenic control of specific traits, somaclonal variation and aneuploidy. Therefore, various biochemical, proteomic and genetic markers have been employed to identify a hybrid progeny of obtained products.

For example, Gleba and Hoffmann (1980) used cytological, isozyme and morphological methods to identify "Arabidobrassica" (*A. thaliana* × *B. rapa*) hybrids. Restriction Fragment Length Polymorphism (RFLP) method was employed for the identification of intertribal somatic hybrids between *B. napus* and *Barbarea vulgaris* by Fahleson *et al.* (1994); Forsberg *et al.* (1994) utilized cytological, RFLP and morphological methods to verify the hybridity of regenerants *B. napus* × *A. thaliana.* Hansen and Earle (1997) used leaf morphology, cytology, and isozyme analyses together with the detection of resistance to fungal pathogen *Alternaria brassicae* to select somatic hybrids between *B. oleracea* and *Sinapis alba.* Two methods, RAPD (Random Amplification of Polymorphic DNA) and the analysis of the protein spectrum by SDS-PAGE (Sodium Dodecyl Sulfate PolyacrylAmide Gel Electrophoresis) were used by Chen *et al.* (2005) to select interspecific somatic hybrids between *B. juncea* and *B. oleracea.* Du *et al.* (2009) employed the combination of cytological analyses, Genomic In Situ Hybridization (GISH) and Amplified Fragment length Polymorphism (AFLP) methods to identify and characterize somatic hybrids between *B. napus* and *Isatis indigotica*; Scholze *et al.* (2010) applied the RAPD analysis and the resistance test against the fungal pathogen *Plasmodiophora brassicae* in various *B. oleracea* somatic hybrids.

CONCLUSIONS

Somatic hybridization through protoplast fusion and the recovery of hybrid plants from protoplast cultures is increasingly being used as a promising component of breeding programmes, aimed at the use of biotechnological methods to create specific materials. Although numbers of somatic hybrids have been obtained by means of protoplast culture technique to date, their adequate utilization in breeding is usually seriously impeded by their poor vitality and reduced fertility. Additionally, only a narrow range of genotypes has good regeneration ability in protoplast cultures.

For the employment of somatic hybridization on a larger scale it is first necessary to optimise the entire procedure of somatic hybridization (protoplast isolation, fusion and regeneration of vigour and fertile plants from protoplast cultures) in genotypes, which have the potential to become initial components to extend the genetic diversity in economically important crops.

Another essential step for effective and routine use of the protoplast culture method in breeding is the identification and selection of fusants with the hybrid progeny. Three fundamental processes and their combinations have been used to fulfil such requirement: (i), identification of heterofusants and their subsequent selection by using a micromanipulator or flow-sorting methods, (ii), controlled survival of only hybrid combinations during regeneration via different selection methods and (iii), identification of somatic hybrids by means of molecular markers. However, none of these procedures is capable to ensure the production of fully "functional" hybrids.

Consequently, in spite of considerable progress and great advances that have been achieved over the past three decades, the method is not yet a routine part of breeding programmes. Therefore, further improvements are necessary for achieving full utilization of protoplast technology also in the breeding of a large group of agricultural crops, belonging to the important family Brassicaceae.

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3.2. The method of protoplast isolation and cultivation in selected Brassicas and the regeneration of whole plants

3.2.1. Kaur N.D., Vyvadilová M., Klíma M., Bechyně M. (2006): A simple procedure for mesophyll protoplast culture and plant regeneration in *Brassica oleracea* L. and *Brassica napus* L. Czech J. Genet. Plant Breed. 42: 103–110.

A Simple Procedure for Mesophyll Protoplast Culture and Plant Regeneration in *Brassica oleracea* L. and *Brassica napus* L.

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Abstract: An improved protocol for *Brassica* protoplast culture and plant regeneration was developed. Isolated protoplasts from four-weeks-old *in vitro* shoot tip culture of *Brassica oleracea* var. *botrytis* cv. Siria F1 and *Brassica napus* doubled haploid of breeding line OP-1 were cultured at a density of 9.8–11.2 × 10^4 protoplasts/ml in darkness at 25°C in a modified medium containing 2% glucose, 0.25 mg/l 2,4-D, 1 mg/l BAP and 1 mg/l NAA. The first divisions of protoplasts were observed on the third day of culture in *B. oleracea* and on the fourth day in *B. napus*. The protoplast cultures were diluted with low osmotic medium on 7th and 11th day. The frequency of dividing cells was about 80% in *B. oleracea* and 50% in *B. napus*. After one month, the microcalli of approximately 0.5–1 mm in size were transferred into an induction medium with various combinations of growth regulators. Minimum duration of enzyme treatment time and extended dark period in the initial phase of culture increased the survival rate of protoplasts. Organogenesis started when the calli enlarged in size on an induction medium (1 mg/l NAA, 0.02 mg/l GA_3, 1 mg/l 2iP) with 2% sucrose and 0.8% agar. Regeneration frequency of calli was found to be 69–75% in *B. oleracea* and 2–3% in *B. napus*. Well-developed shoots were transferred for rooting to a half-strength MS medium without growth regulators. More than 100 *B. oleracea* regenerants were transferred into soil, and they produced normal heads and set seeds. This very simple procedure is efficient and suitable mainly for *B. oleracea* var. *botrytis* and represents a background for fusion experiments.

Keywords: *Brassica napus* L.; *Brassica oleracea* L.; organogenesis; protoplast culture; regeneration

The *Brassica* species being widely used as a vegetable and oilseed crops attracted great attention not only of breeders using conventional methods but also of those concerned with biotechnological methods. Whereas *in vitro* techniques developed in the last decades improved the quality of edible oil (double low cultivars of rapeseed), likewise new oilseed crop varieties useful for industrial purposes were developed (Fahleson *et al.* 1994; Barro *et al.* 2003). Microspore and protoplast culture techniques are used the most frequently for manipulation of foreign genes to broaden the gene pool and in expanding genetic diversity. Protoplasts can be emphasized as good accessories used in crop improvement programmes, particularly in overcoming incompatibility barriers through protoplast fusion (Schenck & Röbbelen 1982; Heath & Earle 1996), development of

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CMS lines (Pelletier *et al.* 1983; Barsby *et al.* 1987; Jourdan *et al.* 1989; Cardi & Earle 1997), genetic transformation through direct uptake of DNA (Spangenberg *et al.* 1986; Maley & Pauls 1985) and mutation breeding. Generation of intergeneric somatic hybrids is no exception, Toriyama *et al.* (1987), Fahleson *et al.* (1994) and Navrátilová *et al.* (1997) have done a great deal of work in this respect.

The regeneration frequencies being genotype dependent vary between species, genotypes and source materials (Bidney *et al.* 1983; Glimelius 1984; Vamling & Glimelius 1990). Plant regeneration has been reported from hypocotyl and leaf protoplasts of *B. oleracea* var. *botrytis* (Glimelius 1984; Jourdan *et al.* 1990; Walters & Earle 1990; Kirti *et al.* 2001) and hypocotyl, mesophyll and stem peel protoplasts (Li & Kohlenbach 1982; Glimelius 1984; Chuong *et al.* 1985, 1987) in *B. napus*. Culture procedures like protoplast embedding in an agarose solidified medium (Barsby *et al.* 1986), and feeder layer cultures (Walters & Earle 1990) used for a small number of protoplasts showed very low regeneration frequencies and were laborious for handling.

The high regeneration ability of protoplasts is a pre-requisite for protoplast utilization in crop improvement programmes. For obtaining a high regeneration frequency, it is necessary to optimize the conditions of protoplast isolation, culture density, composition of culture media and growth regulator concentrations, dark and light periods in the initial culture phase and the developing phase of calli capable of transfer to an induction medium.

The objective of the present work was to improve the protoplast culture and plant regeneration technique in *Brassica napus* and *Brassica oleracea* as a background for their further utilization in fusion experiments.

MATERIALS AND METHODS

Plant material

A pre-screening of plant material for its suitability for protoplast culture included *in vitro* clones of *Brassica napus* microspore-derived doubled haploids, and commercial cultivars of *Brassica oleracea* var. *botrytis* (data not shown). *B. napus* doubled haploid derived from F1 hybrid with double low seed quality originating from the Research Institute of Oilseed Crops in Opava (Kučera *et al.* 2004) and commercial hybrid cultivar Siria F1 of *Brassica oleracea* var. *botrytis* with high embryogenic responsiveness in microspore cultures (Klíma *et al.* 2004) were then selected as model genotypes for detailed experiments. The seeds were germinated after sterilization for one minute in 70% ethanol followed by 30% commercial bleach Savo for twenty minutes on half-strength Murashige and Skoog (1962) medium (MS) without growth regulators in culture cabinets under controlled conditions (with a light intensity of 84 μmol/m^2/s, 16/8 h day/night photoperiod, 23 ± 2°C). Then shoot tips were subcultured continuously on the same medium as donor plant material for protoplast isolation.

Protoplast isolation

Leaves from one-month-old shoot culture were cut transversaly into 1–2 mm segments and treated with 1% cellulase Onozuka R 10 (Serva) and 0.25% macerozyme R 10 (Serva) in W5 salt solution (Menczel *et al.* 1981). The material was kept overnight in a thermostat at 25°C without shaking. Isolated protoplasts were filtered through a nylon mesh (60–72 μm) and transferred into 10 ml centrifuge tubes. W5 salt solution was added to the protoplast suspension and centrifuged at 100 g for 5 minutes. The supernatant was removed and 20% sucrose solution was mixed to the protoplast suspension and covered with W5 salt solution and centrifuged at the same parameters to form a thin ring of floating protoplasts. After centrifugation protoplasts were collected with Pasteur pipette and dispersed in W5 solution for the next two centrifugations. Finally the pellets were dispersed in a culture medium. The number of viable protoplasts was counted after fluorescein diacetate staining according to time intervals of 10–12 h and 16–18 h in the enzyme solution.

Protoplast culture

Protoplasts were cultured at a density of 9.8 to 11.2 × 10^4/ml in 30 mm Petri dishes in 1ml of modified liquid culture medium B (Pelletier *et al.* 1983) supplemented with 0.25 mg/l 2,4-D, 1 mg/l NAA, 1 mg/l BAP and 2% glucose. The cultures were kept at 25°C in dark. After 7 days of the initial culture, 0.5 ml of low osmotic medium C (Pelletier *et al.* 1983) without 2,4-D was added

to each Petri dish and the addition of medium was repeated on the 11th day of culture. Star shaped microcalli developed within 15 days of culture. After the development of microcalli visible by naked eye, the cultures were transferred to light. The plating efficiency defined as the ratio of the number of protoplasts undergoing division to the total number of protoplasts cultured was measured. After about one month when the calli attained sizes of about 0.5–1.0 mm in diameter, they were transferred to solid medium E (PELLETIER *et al.* 1983) at 23°C under dim fluorescent light (40 µmol per m^2/s) in a day/night regime 16/8 h in the culture cabinets. Various growth regulator combinations in medium E (2% sucrose, 1 mg/l NAA, 0.02 mg/l GA_3, 1 mg/l 2iP) were tested, referred to as E_1 (2% sucrose, 0.25 mg/l NAA, 0.02 mg/l GA_3, 1 mg/l 2iP, 100 ml/l coconut milk, 4 g/l agarose), E_2 (2% sucrose, 3.0 mg/l BAP, 0.1 mg/l GA_3, 4 g/l agarose) and E_3 (1 mg/l NAA, 0.02 GA_3, 1 mg/l BAP).

Plant regeneration

The percentage of callus formation frequency was calculated as the number of formed calli to the total number of protoplasts undergone division. The well developed calli with shoot primordia were transferred to regeneration media F (0.5 mg/l BAP, 0.1 mg/l NAA, 1% sucrose, 8 g/l agar) and MS without growth regulators with 1% sucrose and 10 g/l agar. Regeneration frequency was established as the ratio of the number of calli regenerating shoots to the number of calli developed in the set experiment.

Chromosome analysis

The ploidy level of regenerants was assessed according to the flower morphology, seed set and chromosome counts. Chromosomes were counted in root tip cells. When the roots were about one centimetre in length, they were treated with paradichlorobenzene for 3 h, hydrolyzed in 1N HCl (15 min at 60°C) and fixed in ethylalcohol and acetic acid (2:1). The roots were squashed in a drop of aceto-orcein and observed under 1000× magnification.

RESULTS

A marked quantitative difference of more than 19% in viability (Table 1) of protoplasts was observed between the two time regimes in the enzymatic solution (10–12 h, 16–18 h). Three types of protoplasts were found in the isolated cultures, i.e. small, medium and large ones (Figure 1). Divisions occurred in all the three types of protoplasts and started on the 3rd day of culture in *B. oleracea* and on the 4th day of culture in *B. napus* (Figure 2). On the 7th day of culture more than two mitotic divisions were observed in 60% of protoplasts in *B. oleracea*, but in *B. napus*, the frequency was low (30%). After the addition of medium C (PELLETIER *et al.* 1983), the division frequency increased and microcolonies of 8–16 cells were observed in 80% of protoplasts in *B. oleracea*, whereas in *B. napus* microcolony formation was found in less than 50% of protoplasts. The high division frequency after the addition of low osmotic solution indi-

Table 1. Effect of enzyme treatment duration on viability of protoplasts

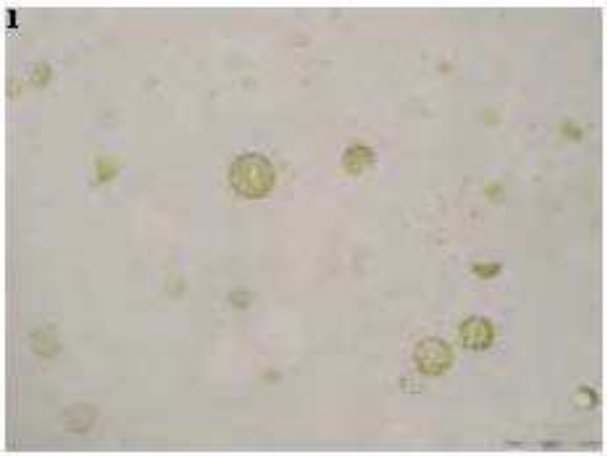
Duration of enzyme treatment	No. of protoplasts (MFA)	No. of viable protoplasts	Viable protoplasts (%)
10–12 h	14.1	6.9	48.9
16–18 h	13.4	4.0	29.9

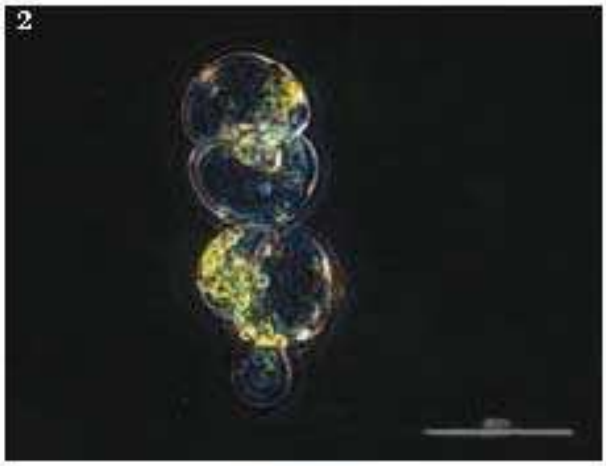
MFA – Microscopic Field Area

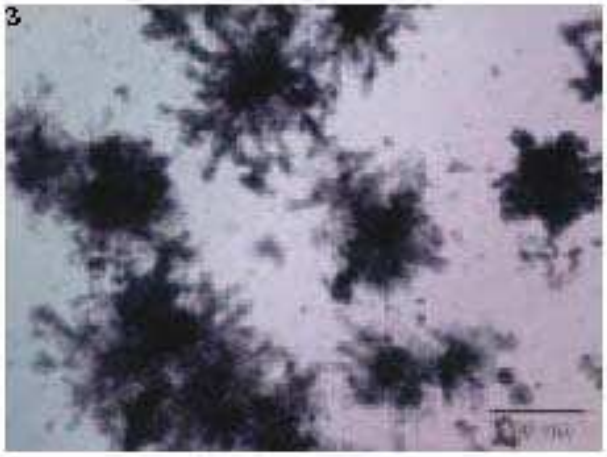
Table 2. Regeneration in a mesophyll protoplast culture of *B. oleracea* var. *botrytis* cv. Siria and *B. napus* breeding line OP-1

Species	Culture density	Plating efficiency (%)	Callus formation frequency (%)	Percentage of regeneration (%)
B. oleracea	11.2×10^4/ml	80.87 ± 4.96	47.99 ± 7.80	69.05 ± 7.45
B. napus	9.8×10^4/ml	47.94 ± 7.43	39.96 ± 5.31	2.72 ± 2.98

Data (means and standard deviation) show results from two independent experiments with each species







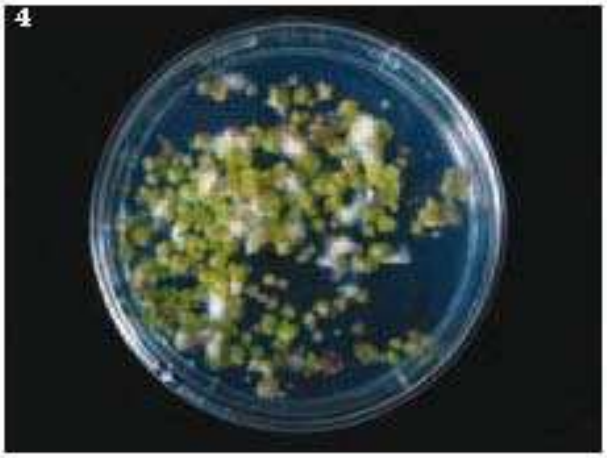


Figure 1. Freshly isolated protoplasts of *B. oleracea* (bar = 100 µm)

Figure 2. The first division after four days of protoplast culture in *B. napus* (bar = 50 µm)

Figure 3. Star shaped microcalli from *B. oleracea* protoplasts after 15 days in culture (bar = 500 µm)

Figure 4. Green calli after about three weeks in an induction medium (*B. oleracea*); the calli bear root hair-like outgrowths and shoot buds

cates that a higher concentration of osmoticum, beneficial in the initial cultures, inhibits enhanced cell division essential for proliferation of microcalli. The microcolonies enlarged and became star shaped (Figure 3) within 15 days of culture. Based on different experiments, it is concluded that the time used for isolation of protoplasts in the enzyme solution represented a crucial factor. The protoplasts isolated within 10–12 h showed higher viability than those isolated within 16–18 h. Temperature and light have a profound effect on division frequency. The cultures which were transferred after 7 days to light at 23°C showed very few divisions in comparison with those kept in dark for 15 days at 25°C. The developmental stage of calli in the liquid culture is important for transfer onto the solid medium. In our experiments one-month-old calli reached the size of about 1 mm and after transferring to medium E, within 2–3 weeks they enlarged in size and showed a good regeneration ability. Hair-like root overgrowths appeared on calli very often and later shoot bud primordia originated on the same calli. In the initial developmental phase (1–2 weeks) the calli were cream-yellow in colour but after a period of two weeks they turned green (Figure 4). In *B. oleracea* the calli were of fragile consistency and contained embryo-like structures; when these structures were transferred to the regeneration medium, they regenerated shoots. The calli that did not regenerate on medium E readily regenerated shoots on medium F (PELLETIER *et al.* 1983)

Figure 5. Plant regenerated from the protoplast derived callus in *B. oleracea* developing roots

or MS without growth regulators. Medium E is essential for the induction of shoot regeneration in developing calli. The shoots were transferred to half-strength MS medium for rooting and for prevention of vitrification (Figure 5). In *B. oleracea* all the developed shoots regenerated into whole plants whereas in *B. napus* the plant regeneration efficiency was 45–50%.

From various modifications of induction medium, media E and E1 produced regeneration frequencies of about 70% in *B. oleracea* but in the case of *B. napus* the regeneration frequency was very low (3%). Medium E2 induced stem-like structures in the calli of *B. oleracea* and regenerated shoots after further transfer to the same medium. The higher concentration of BAP seemed to lead to the formation of embryo-like structures. The development of shoots took comparatively a longer time and regeneration frequency was about 31%. In *B. napus* the calli enlarged but no regeneration took place. In medium E3 calli regenerated shoots in *B. oleracea* but the percentage was lower than 15% (Table 3). Several independent experiments were performed for cauliflower cultivar Siria F1 and rapeseed breeding line OP-1. Table 2 details the development of cultured protoplasts of both species. Morphological and horticultural characteristics of about hundred regenerants of *B. oleracea* grown in the greenhouse were examined. The majority of the plants were normal, but some plants with morphological alterations such as absence of apical meristem or curly small leaves were recorded (Figure 6). All the plants developed normal white curds (Figure 7) except two that had violet streaks on their heads. Flowers were normal but large variation in the production of pollen was noted. Most of the plants had flowers with a good amount of pollen. All the examined *B. oleracea* regenerants had the expected $2n = 18$ chromosome number.

DISCUSSION

The regeneration of plants from protoplasts is a prerequisite for their utilization in crop improve-

Figure 6. *B. oleracea* abnormal plant bearing curly leaves

Figure 7. *B. oleracea* plant generated from protoplast with normal head

Table 3. Effect of different media on shoot regeneration from protoplast derived calli in *Brassica oleracea* var. *botrytis* cv. Siria

Medium**	No. of cultured calli	No. of survived calli	No. of calli with shoots	Regeneration (%)
E	327	327	225	68.80
E_1	280	280	177	63.20
E_2	202	193	59*	30.56
E_3	107	99	14	14.14

*The calli developed into stem-like structures, the small axes were subcultured and developed into shoots. A total of 59 plants were regenerated out of 193 calli

**E – 2% sucrose, 1 mg/l NAA, 0.02 mg/l GA3, 1mg/l 2iP; E_1 – 2% sucrose, 0.25 mg/l NAA, 0.02 mg/l GA3, 1 mg/l 2iP, 100 ml/l coconut milk, 4 g agarose; E_2 – 2% sucrose, 3.0 mg/l BAP, 0.1 mg/l GA3, 4 g agarose; E_3 – 1 mg/l NAA, 0.02 GA3, 1 mg/l BAP

ment programs. *Brassica oleracea* var. *botrytis* cv. Siria being an important vegetable and having higher protoplast division efficiency (80%) and regeneration ability (70%) can easily be used in crop improvement practices. Minimum duration of enzyme treatment time and extended dark period in the initial phase of culture increased the survival rate of protoplasts and hence the regeneration process. In the previous findings in *Brassica* vegetables, either the survival rate was low or the regeneration percentage decreased (Glimelius 1984; Kirti *et al.* 2001). The protocol proposed by us is simple, rapid and effective.

Regenerants from both *B. oleracea* and *B. napus* were successfully produced using the improved culture procedure. The isolation time of protoplasts in an enzyme solution greatly affects not only the viability of protoplasts but also the nature of plasmalemma (Pilet 1985), thus affecting the wall biosynthesis and hence the division process. Culture density has a profound effect on the plating efficiency of protoplasts. In concordance with the results of Chuong *et al.* (1985), it was found that with higher culture densities higher plating efficiency could be achieved. For mesophyll protoplasts, Vamling and Glimelius (1990) also recommended a higher concentration of protoplasts.

In the initial culture medium, an equal amount of BAP and NAA in the presence of a low amount of 2, 4-D was favourable in our experiments. Dietert *et al.* (1982) also pointed to the better growth of callus in the presence of a low concentration of 2,4-D. However, according to Glimelius (1984) and Kohlenbach *et al.* (1982) a high amount of 2, 4-D is essential for cell division and callus proliferation. No such effect was evident in our experiments. It might be linked to the endogenous level of auxins in the used plant material.

A prolonged dark period is essential for the stability of protoplasts and hence for the formation of microcalli. In the presence of light, H^+ ion extrusion takes place, which increases the acidity of culture medium (Schubert & Matzke 1985). Cleland (1975) showed that H^+ ion extrusion was enhanced by the action of auxins. Under illumination the cultures turn brown, which affects the division efficiency of protoplasts. In our experiments not even after one month of culture was there a sign of browning in the cultures which were transferred to light after microcalli had been formed.

Differentiation or induction medium is a critical part of the protoplast culture protocol. Based on our results it can be concluded that cytokinin 2iP is more efficient than BAP in the induction of regeneration of calli. After one month on the induction medium, further transfer to regeneration medium F and MS (without growth regulators) showed no profound effect on regeneration frequency. The differentiation of organs was found to take place on medium E. Development of fragile calluses is in accordance with the reports by Kirti and Chopra (1990) in *B. juncea* and Kohlenbach *et al.* (1982) in *B. napus*. The absence of root development in almost 50% of regenerated shoots in *B. napus* is comparable to the results of Qiong Hu *et al.* (1999) and is genotype dependent. In our experiments, *B. oleracea* and *B. napus* showed a high division frequency in medium B in comparison with medium KM8p (Kao & Michayluk 1975),

which corresponded to protoplast division in some haploid lines generated from a microspore culture of *B. carinata*. In the present liquid culture system it is easy to handle the calli, and the medium described by PELLETIER *et al.* (1983) proved to be quite suitable for *B. oleracea* protoplast culture. There are some variations in growth regulator concentration requirements for different subspecies (ROBERTSON *et al.* 1988; JOURDAN *et al.* 1990; KIRTI *et al.* 2001).

Mesophyll protoplasts have several advantages over the hypocotyl ones. A large number of protoplasts can be obtained from just one plant, and further offspring can be regenerated from apical meristems in subsequent culture cycles and used for protoplast isolation. Further, mesophyll protoplasts are preferable for elite lines for which enough seed is not available, whereas for hypocotyls a huge amount of seeds with good germination is required. Mesophyll protoplasts can be used as a very effective material in overcoming incompatibility barriers through somatic hybridization, genetic manipulation, for studying cell metabolism and generation of genetic variation in a short time period.

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Abbreviations

BAP – 6-benzylamino purine
2,4-D – 2,4-dichlorophenoxyacetic acid
GA_3 – gibberellic acid
2iP – isopentenyl adenine
NAA – 1-naphthalene acetic acid

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3.3. Chemical fusion and electrofusion in selected genotypes and the regeneration after fusion in protoplast culture

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PROTOPLAST ISOLATION AND FUSION BETWEEN *BRASSICA CARINATA* BRAUN. AND *BRASSICA RAPA* L.

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Abstract

Brassica carinata mesophyll and B. rapa hypocotyl protoplasts were used for fusing experiments using polyethylene glycol (PEG). Treatment with 30% PEG 6000 MW for 10 minutes was more effective, while combinations with 30% PEG 6000 MW/15 min, 30% PEG 4000 MW/15 min and 30% PEG 4000 MW for 10 minutes reduced viability of protoplasts and cell wall regeneration, cell division and production of microcolonies. Fusion frequency was about 25% with the treatment of 30% PEG 6000 MW/10 min. Cell colonies forming callus was observed after 14 days of cultivation on liquid B medium with addition of liquid C medium. Microcalli were subsequently transferred to solid E and F medium. Shoot regeneration from calli was induced via indirect organogenesis and somatic embryogenesis on MS hormon free medium. In total 58 calli were obtained from fusions between B. rapa 31/96 and B. carinata line 1; 14 calli produced shoots. According to the morfological diferencies half of shoots were heterocaryonicals. This observation approved the events from Flow cytometry. Approximately 40% of analyzed samples were only B. carinata plant type established via protoplasts fusion, 60% were of B. carinata and B. rapa.

Key words: PEG, protoplast, Brassica, fusion
Abbreviations: PEG, Polyethylene glycol; MS, Murashige and Skoog (1962)

INTRODUCTION

Genus *Brassica* is the most economically important one within the family *Brassicaceae* (syn. *Cruciferae*). Many species and types of Brassicas are fundamental oilseed crops, vegetables, forage crops, and are used for the production of spices, such as mustard. Among the *Brassica* crops, oilseeds have the highest economic value. *B. carinata* Br (2n = 17 BBCC), also known as Abyssinian mustard (Bajaj, Mohapatra, 1987) is a natural amphidiploid (Narasinhulu, Kirti, et al. 1992, Jourdan, Salazar, et al., 1992) with significant fungal resistance (Choudhari, Joshi, Ramarao, 2000) water stress (Narasinhulu, Kirti, 1992) and aphid attack tolerance (Yang, Jia, et al. 1990, Babic, Datla, et al., 1998). *B. carinata* plays an important role as an oilseed crop especially in tropical and subtropical regions but the quality of oil is lower in comparison with *B. rapa L.*

Protoplast fusion allows the creation of hybrid and cybrid combinations of species that are sexually incompatible, thus facilitating the transfer of genes from a related, but sexually incompatible species, to another one without genetic transformation. This technology has allowed not only intrageneric hybridizations, but the production of intergeneric hybrids and cybrids as well. Various desirable traits from the parents have been transferred to the hybrids and cybrids using this technology. In this study new pathway for breeding of *B. carinata* and *B. rapa* species was founded. Protoplast fusion was used as an effective tool for breeding brassicas. The target was to obtain optimal methods *in vitro* for breeding Brassicas. Considerable progress was accomplished in the cellular and molecular biology of Brassica species in the past few years. Plant regeneration has been increasingly optimized via organogenesis and somatic embryogenesis by means of various explants; with tissue culture improvements focused on factors such as age of the explant, genotype, and media additives. Somatic cell fusion has facilitated the development of interspecific and intergeneric hybrids in the sexually incompatible species of Brassica (Cardoza, Stewart, 2004). The aim of this study was to verify and optimize current methods for establishing protoplast culture in selected genotypes of genus *Brassica.* Consequently, fusion experiments by means of PEG solution between *B. rapa* and *B. carinata* have been made to obtain new genotype combinations for subsequent breeding processes.

MATERIALS AND METHODS

Four genotypes of *B. carinata* (lines 1, 2, 3, and yellow-seeded line 15H2901003) and nine genotypes of *B. rapa* (cultivars Reward, Tobin, Eldorado, Candle and lines P3/86, 33/96, 31/95, 31/96 and line 6 with quadruple pods) from the Czech National Gene Bank in the Crop Research Institute and yellow seeds from the Czech University of Agriculture Prague, Institute of Tropics and Subtropics were used as the donor plants for protoplast culture.

Donor plants

Seeds were surface sterilized in 70% ethanol (1 minute) and then in 30% NaOCl (commercial bleach SAVO) for 20 minutes. Then were rinsed three times in sterile distilled water and sown on MS medium without growth regulators (Hu, Andersen, Hansen, 1999). Plant material for mesophyll protoplasts was cultivated in a cultivation room under controlled environmental conditions (25°C and 16/8h day/night photoperiod). Seedlings for etioled hypocotyl protoplasts were incubated in a thermostat at 25°C in the dark.

Protoplast isolation and cultivation

Approx. 25 young leaves from 28 days old plant and 30 etiolated hypocotyls from 7 days old seedlings were segmented to 2mm slices and placed separately to 60mm plastic Petri dishes with 5 ml of an enzymatic solution (mixture of 1% Cellulase Onozuka R-10 and 0,25% Macerozyme in W5 medium (Mukhopadhyay, Arumugan, Pradhan, Murthy, Yadav, Sodhi, Pental, 1994), put into the thermostat and incubated at 25°C for 18 hours in the dark.

Protoplast suspension was purified via repeated (three times) centrifugation in W5 medium (5 minutes at 100 G), supernatant removal, rewashing and subsequent centrifugation on sucrose gradient (Pelletier et al., 1993).

Protoplast fusion

Purified protoplasts were resuspended in 0,1 – 0,5 ml M+C medium, mesophyll and hypocotyl protoplasts were then mixed in the ratio of 1:1 and the density was adjusted to $10^5 – 10^6$ protoplasts per 1 ml. 4 x 50 µl (4 drops) of protoplast suspension was added to the 35mm Petri dish and sedimented for 20 minutes. 50 µl of PEG 6000 or 4000 MW (Molecular weight) was gently added to each drop of sedimented protoplasts and incubated for 10 or 15 min. Then the PEG was carefully removed and 200 µl of STOP solution was added to each drop After 20 minutes of incubation the STOP solution was replaced by B liquid cultivation medium. Petri dishes with protoplasts were transferred to the thermostat and cultivated at 25°C in the dark for 3 days then cultivated according to Pelletier et al. (1993).

Plant regeneration

Cell wall was established spontaneously 7 days after protoplasts fusion according to microscope observation. Cell microcolonies were produced on liquid B medium with addition of liquid C medium after 14 days. Well developed microcalli were then transferred onto solid E medium. After 14 days of cultivation calli were placed onto solid F medium. Shoots, regenerated from calli via indirect organogenesis and somatic embryogenesis on F medium, were transplanted onto rooting MS hormone-free medium and were cultivated in a cultivation room under controlled environmental conditions (25°C and 16/8h day/night photoperiod). Plantlets with well developed roots were transferred to *in vivo*.

Fig. 1– callus culture and regeneration of *B. rapa* 31/96 and *B. carinata* line 1

Plant selection

Flow cytometry procedure using Otto I+II buffers

1. Fluorochrome (DAPI, 4 µg/ml or propidium iodide + RNase, both 50 µg/ml) were added to the selected buffer; ß-mercaptoethanol (2 µl/ml) might be used to avoid polyphenolics oxidation.

   (DAPI-staining: 25 ml Otto II buffer + 1 ml DAPI stock solution + 50 µl ß-mercaptoethanol; Propidium iodide-staining: 20 ml Otto II buffer + 1 ml PI stock solution + 1 ml RNase stock solution + 40 µl ß-mercaptoethanol).

2. Young intact leaf tissues (typically 1 cm^2) were chopped with a new razor blade in a Petri dish containing 1 ml of ice-cold Otto I buffer.

3. Filter the suspension through a 42 µm nylon mesh.

4. Analyse relative DNA content of isolated nuclei (Doležel, Binárová, Lucretti, 1989, Matzk, Meister, Schubert, 2000, Pfosser, Amon, Lelley, Heberle-Bors, 1985).

Otto Buffer I content in 200 ml solution: 4.2 g of 0.1 M citric acid monohydrate and 1 ml of 0.5% (v/v) Tween 20. Otto Buffer II content in 200 ml solution: 28.65 g of 0.4M $Na_2HPO_4 .12H_2O$ (Otto, 1990).

RESULTS AND DISCUSSION

Activity of PEG 6000 MW/10 min. *Brassica carinata.*

Genotype	Cell division	Microcolonies	Callus	Regeneration via indirect organogenesis	Regeneration via somatic embryogenesis
15H2901003	+	+	-	-	-
Line 1	+	+	+	+	+
Line 2	-	-	-	-	-
Line 3	+	+	+	-	-

+ = positive result
- = negative result

Activity of PEG 6000 MW/10 min. *Brassica rapa* subsp. *oleifera* f. *praecox*, f. *biennis.*

Genotype	Cell division	Microcolonies	Callus	Regeneration via indirect organogenesis	Regeneration via somatic embryogenesis
Reward	+	-	-	-	-
Tobin	+	+	+	-	-
Eldorado	+	-	-	-	-
Candle	+	+	+	-	-
P3/86	+	-	-	-	-
Line 33/96	+	+	-	-	-
Line 31/95	-	-	-	-	-
Line 31/96	+	+	+	+	+
Line 6	-	-	-	-	-

+ = positive result
- = negative result
Other results (activity of different MW of PEG and time of activity) are described bellow.

The development of protoplast technology for *B. rapa* and *B. carinata* has permitted the production of somatic hybrids between these cruciferous species and the formation novel cybrids and hybrids (Jaiswal, Hammat, Bhojwani, Cocking, Davey, 1990). 58 calli and 14 calli produced shoots by protoplasts fusion between *B. rapa* 31/96 and *B. carinata* line 1. Fusion frequency was about 25%. The differentiation process and shoot regeneration took 3-4 months; similar results were published before (Fahleson, Eriksson, Landgren, Stymmne, Glimelius, 1994b, Gerdemann-Knörck, Nielen, Tzscheetzsch, Iglisch, Schieder, 1995). Fusion and regeneration frequencies were almost the same as those obtained in hybridization experiments between more closely related species (Fahleson, Eriksson, Glimelius, 1993). The three parameters of protoplasts viability, yield and freqeuncy of cell division were used for arriving at the optimum MW of PEG and time of activity. This is more important for identification of suitable MW and time of activity influencing yield and viable protoplasts, for plant breeding Brassicas especially *B. carinata* Br. and *B. rapa* L. It is optimal for protoplast fusion, using 30% PEG 6000 MW per 10 min. 30% PEG 6000 MW/10 min solution was more effective, while combinations with 30% PEG 6000 MW/15 min, 30% PEG 4000 MW/15 min and 30% PEG 4000 MW for 10 minutes reduced viability of protoplasts and cell wall regeneration, cell division and production of microcolonies. These results conform to previously published work (Kirti, Prakash and Chopra, 1991, Kirti, Narasimhulu, Prakash and Chopra, 1992 a, b, Narasinhulu, Parkash, Chopra 1992). According to the morfological diferencies, one half of shoots were heterocaryonicals. One part of regenerated plantlets remined plants of *B. carinata* (from the aspect of size and form of leaves) and second part did not reminde *B. carinata* nor *B. rapa*. This observation approved the events from Flow cytometry. The obtained details proved that approximately 40% analyzed samples were only *B. carinata* plant established by via fusion of

protoplasts within this species and 60% of plantlets established via protoplast fusion between *B. carinata* and *B. rapa*. Flow cytometry analyses data are shown in Fig. 2, 3 and 4. Current results confirm that the successful and efficient utilization of tissue culture technique depends not only on species but even on variety (Power, Chapman, Wilson, 1985, Moreno-Ferrero, Nuez-Viñals, 1985, Li, Stoutjestijk, Larkin, 1999, Jain, Chowdhury, et al. 1988). According to our results, both *B. rapa* - 31/96 and *B. carinata* line 1 are suitable genotypes for further experiments in protoplast fusion. Successful plant regeneration from other varieties of *B. carinata* and *B. rapa* was not gained.

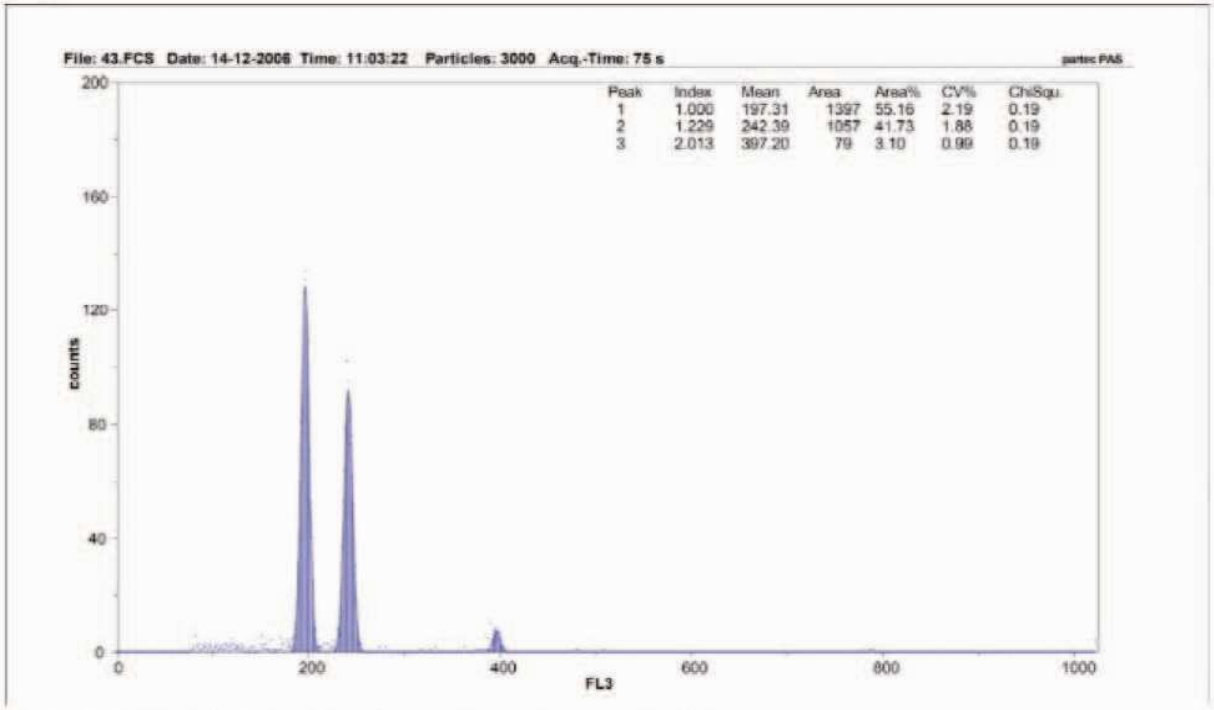

Fig. 2. : Histogam of Flow cytometry of *Brassica carinata* genotyp 1

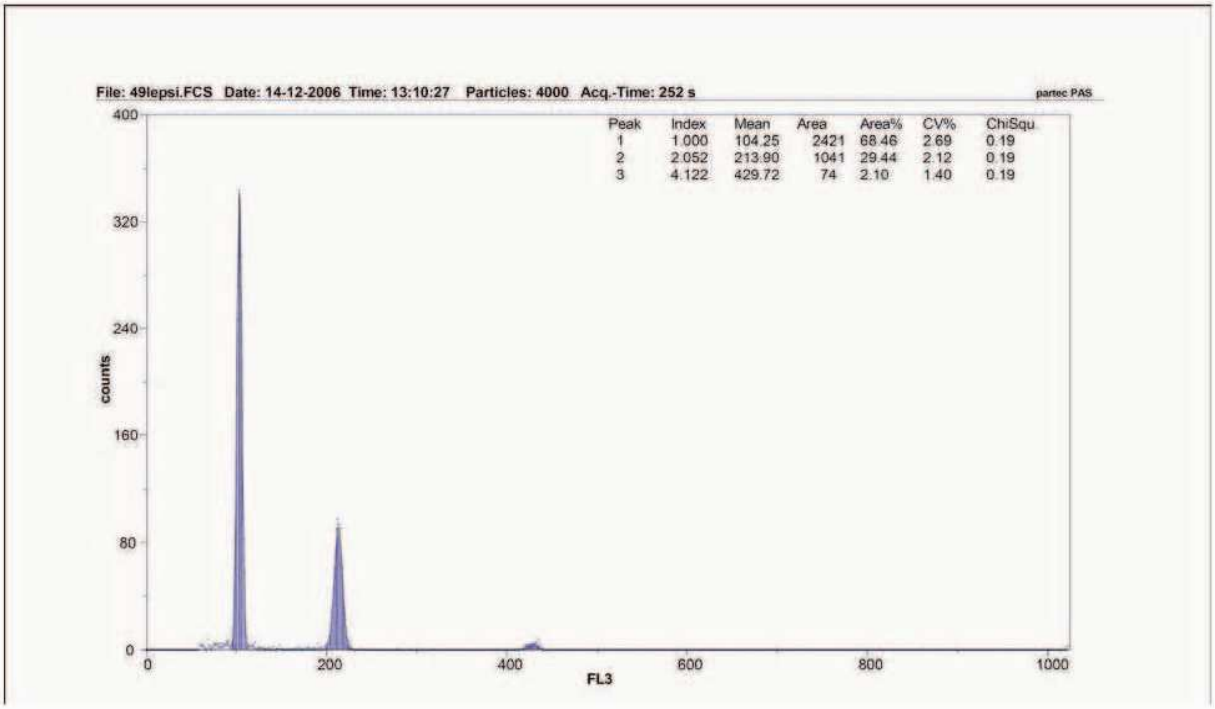

Fig. 3. : Histogam of Flow cytometry of *Brassica rapa* subsp. *oleifera* f. *praecox* 31/96

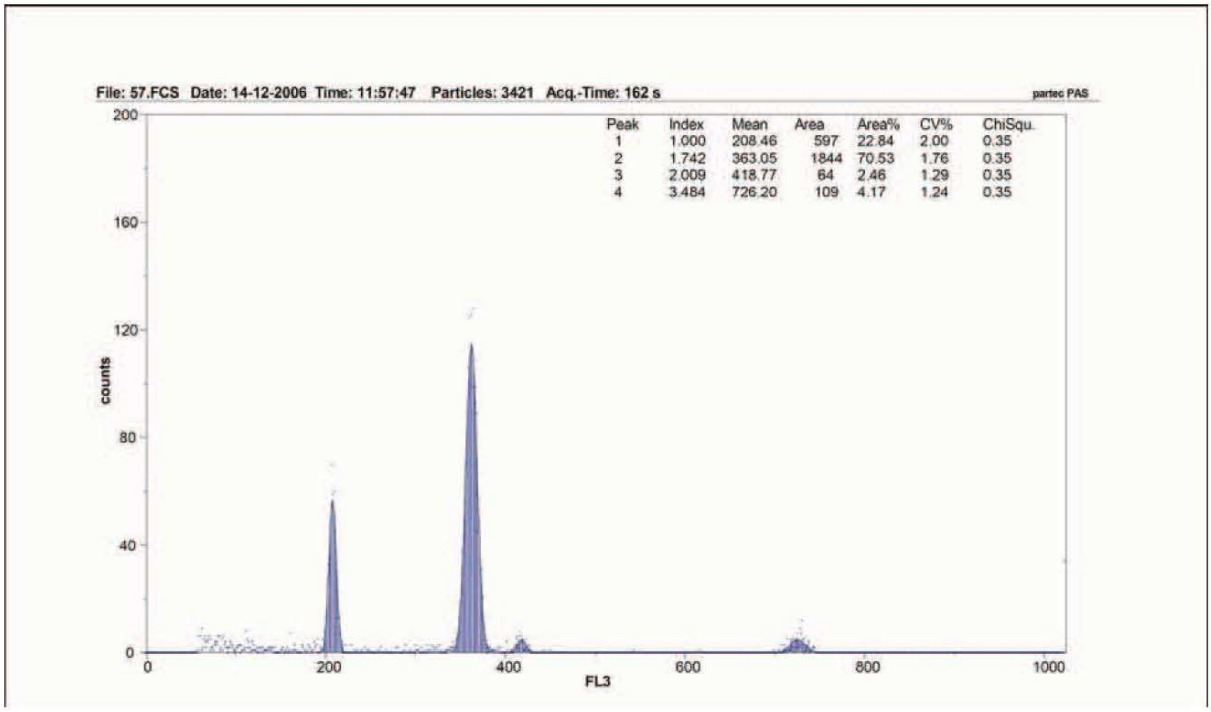

Fig. 4. : Histogam of Flow cytometry of *Brassica rapa* subsp. *oleifera* f. *praecox* 31/96 x *Brassica carinata* genotyp 1

Fig. 5. : Regeneration genotypes B. r. 31/96 and B. c. 1

Fig. 6. : Regeneration genotypes B. r. 31/96 and B. c. 1 via indirect organogenesis

Fig. 7, 8. : Regeneration genotypes B. r. 31/96 and B. c. 1 via somatic embryogenesis

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ELEKTROFÚZE A CHEMICKÉ FÚZE PROTOPLASTŮ VYBRANÝCH DRUHŮ RODU *SOLANUM, CUCUMIS A BRASSICA.*

ELECTROFUSION AND CHEMICAL FUSION OF PROTOPLASTS IN SELECTED SPECIES OF GENUS *SOLANUM, CUCUMIS* AND *BRASSICA.*

Marie GREPLOVÁ, Božena NAVRÁTILOVÁ, Miroslava VYVADILOVÁ, Miroslav KLÍMA

Abstrakt

Protoplast fusions give rise to formation of unique mixtures of genetic information – transfer of nuclear and cytoplasmic genetic information between plant species, genera that could not be derived from classical crossing and it offers avoiding of sexual barriers e.g. in plant breeding. The aim of protoplast fusion is transfer of genes controlling certain feature e.g. from wild-grown plant into agronomic important crop. Characteristic effort of experiments was and is introduction of resistance genes to various viral and fungal diseases form wild plant species, cytoplasmic male sterility (CMS), resistance to stress, incl. salt-tolerance and low-temperature tolerance, resistance to insect pests (phytoalexin synthesis), storage protein gene synthesis, genes for vitamin synthesis.

The aim of this study is using protoplast electrofusion and chemical fusions for somatic hybridization, especially for increasing of genetic variability in selected important vegetable species of Brassica, Cucumis and Solanum genus and possibility of including this method into breeding approaches to obtain new breeding materials. Mesophyll protoplasts were isolated from in vitro cultured plants of Armoracia rusticana, Brassica olearacea (B. oleracea var. capitata, B. oleracea var. botrytis), Cucumis metuliferus and Solanum tuberosum dihaploid and tetraploid plants and wild Solanum species (S. brevidens, S.berthaultii, S. bulbocastanum, S. pinnatisectum, S.verrucosum). Hypocotyl protoplasts were isolated from etiolated seedlings of Brassica oleracea (var. botrytis, var. capitata) and callus protoplasts of Cucumis melo. Enzymatic mixture of 1 % or 2% Cellulase Onozuka R-10 and 0,25 % or 1 % Macerozyme R-10 in rinsing solution Pgly/W5 (3,2) was used for isolation of mesophyll and hypocotyl protoplasts or callus protoplasts. Protoplasts were isolated using a conventional method (filtration, centrifugation and purification with 20 % sucrose gradient). Viability of isolated protoplasts used for electrofusions and chemical fusions was more than 80 % (determined using FDA). Fusing partner components were mixed in the ratio of 1:1. There were fused protoplasts of Brassica genus: mesophyll and hypocotyl, Cucumis genus: mesophyll and callus, Solanum genus: mesophyll and mesophyll. Protoplast electrofusion was performed using apparatus ECM 2001 and in electroporation chamber for 20-30 µl with the 0,5 mm electrode distance. Following parameters were used for fusions: for protoplast alignment – 5 V AC and 1 MHz frequency with individual duration of action, for fusion - 1 pulse 10 V DC of length 80 µs (4). Regeneration of cell walls was recorded and first division was found in 6-8 days of culture in all cases, only in Solanum genus microcolonies were found in 15 days and start of plant regeneration (organogenezis) were found in 140 days. The experiments have not been finished yet. Following electrofusions protoplasts were viable and undamaged. Homofusion and heterofusion products and also unfused protoplasts could be observed. Chemical fusion was performed using polyethyleneglycol (33 % PEG) for 15 min. After fusion the protoplasts appeared intact and surviving. The hybrid products of vision (mesophyll + hypocotyl, mesophyll + callus) contained rich vacuole system and many chloroplasts, i.e. characters of both fusion partners. The somatic hybridization by chemical fusion between Brassica + Brassica and Brassica + Armoracia lead to production of regenerated plants but between Cucumis metuliferus and Cucumis melo only to regenerated microcalli. The somatic hybridization by chemical fusion between Solanum tuberosum and wild Solanum species lead to regeneration cell walls and first cell divisions.

Key words: electrofusion, chemical fusion, polyethylene glycol, protoplast, somatic hybridization, Brassica, Cucumis, Solanum

Úvod

Fúze protoplastů vedou ke vzniku jedinečných směsí genetických informací - přenos jaderné a cytoplazmatické genetické informace mezi rostlinnými druhy, rody, které by nebylo možné získat při klasickém křížení a nabízí překlenutí sexuálních bariér např. ve šlechtění rostlin. Cílem fúzí protoplastů je přenos genů kontrolujících určitý rys např. z planě rostoucí rostliny do hospodářsky významné plodiny. Charakteristickou snahou pokusů s fúzí protoplastů bylo a je vnášet:

- geny rezistence vůči různým virovým a houbovým chorobám z planých druhů rostlin
- cytoplazmatickou samčí sterilitu (CMS)
- rezistenci ke stresům, včetně tolerance k zasolení, chladu
- rezistenci vůči hmyzím škůdcům (syntéza fytoalexinů)
- geny pro syntézu zásobních proteinů, vitamínů.

Cílem předkládané práce je využití elektrofúzí a chemických fúzí protoplastů pomocí PEG v somatické hybridizaci za účelem zvýšení genetické variability u vybraných zástupců rodu *Solanum, Cucumis a Brassica* a možnost zařazení těchto metod do šlechtění zemědělských plodin s cílem získat nový šlechtitelský materiál.

U rodu *Solanum* a *Brassica* byly realizovány úspěšné fúze již na počátku 80 let minulého století. Byl získán šlechtitelský materiál se zvýšenou rezistencí k některým chorobám u *Solanum* např. vůči *Phytophtora infestans*

(Thieme et al. 1997) (10), u rodu *Brassica* rezistence vůči *Alternaria* spp. (Hansen a Earle 1997) (8), vůči *Xanthomonas campestris* (Hansen a Earle 1995) (8) nebo přenos samčí sterility - CMS (Sigareva a Earle 1997) (8). U rodu *Cucumis* byly výsledky metody elektofúzí nebo chemických fúzí zatím méně úspěšné, regenerace se zastavila ve fázi mikrokalusů nebo rostliny odumíraly (Gajdová et al., 2004) (5).

Materiál a metody

Rostlinný material

Rostliny *S. tuberosum* (dihaploidní a tetraploidní rostliny) a diploidní plané druhy rodu *Solanum* (*S. brevidens, S. berthaultii, S. bulbocastanum, S. pinnatisectum* a *S. verrucosum)* byly kultivovány *in vitro* na SH mediu s přídavkem 3 mg/l $AgNO_3$ a 1,5 mg/l Alaru 85 jako zdroj mezofylových protoplastů.

Rostliny *Brassica oleracea* var. b*otrytis, capitata a Armoracia rusticana* byly kultivovány *in vitro* na MS mediu jako zdroj mezofylových protoplastů (jen plně vyvinuté mladé listy) a etiolované hypokotyly rodu *Brassica* byly získány z *in vitro* ve tmě naklíčených 7-denních semenáčků jako zdroj hypokotylových protoplastů.

Rostliny *Cucumis* m*etuliferus* byly kultivovány *in vitro* na MS mediu jako zdroj mezofylových protoplastů a 7-týdenní kalusy odvozené ze segmentů listů *Cucumis melo* na médiu MS doplněném 2,5 mg/l NAA a 1,0 mg/l BAP jako zdroj kalusových protoplastů.

Izolace protoplastů

Pro izolaci mezofylových a hypokotylových protoplastů byla použita směs enzymů (1 % Cellulase Onozuka R-10 and 0,25 % Macerozyme R-10) rozpuštěných v promývacím roztoku W5 (*Solanum, Brassica*) (2) nebo Pgly (*Cucumis*) (3). K izolaci protoplastů z kalusu (*Cucumis melo*) byla použita směs enzymů o vyšší koncentraci (2 % Cellulase Onozuka R-10 and 1 % Macerozyme R-10) v promývacím roztoku Pgly (3). Listy, hypokotyly nebo kalusy byly nakrájeny na malé segmenty a ponořeny do ezymatického roztoku do termostatu při teplotě 25 °C na dobu 16-18 hodin. Protoplasty byly izolovány konvenční metodou (filtrace přes nylonové sítko – velikost pórů 72 μm, centrifugace v promývacích roztocích a separace s pomocí 20 % sacharosového gradientu). Životnost purifikovaných protoplastů byla nad 80 % (hodnoceno pomocí FDA).

Fúze protoplastů elektrickým polem

Purifikované protoplasty byly promyty přefúzním roztokem (0,4 M manitol a 1 mM $CaCl_2$) a poté fúzním roztokem (0,4 M manitol). Byla upravena jejich hustota (10^5 – 10^6 protoplastů na 1 ml). Protoplasty ve zvolených kombinacích byly smíchány v poměru 1:1. U rodu *Solanum* byly fúzovány protoplasty mezofyl – mezofyl, u rodu *Brassica* mezofyl – hypokotyl, u rodu *Cucumis* mezofyl – kalus. Fúze byly realizovány pomocí přístroje ECM 2001 v elektroporační komůrce o objemu 20-30 μl se vzdáleností elektrod 0,5 mm. Byly použity následující parametry elektrického pole: pro seřazení protoplastů do řetízků (obr.1) 5 V střídavého proudu při frekvenci 1 MHz s individuální dobou působení v závislosti na kvalitě vzorku a hustotě protoplastové suspenze. Pro vlastní fúzi protoplastů elektrickým polem byl použit 1 puls stejnosměrného proudu o velikosti 10 V s dobou trvání 80 μs. Aktivita protoplastů v elektrickém poli byla pozorována v inverzním mikroskopu.

Chemická fúze protoplastů

Purifikované mezofylové protoplasty (*Brassica, Armoracia*) byly fúzovány s hypokotylovými protoplasty (*Brassica*) nebo mezofylové protoplasty (*Cucumis metuliferus*) s kalusovými protoplasty (*Cucumis melo*). U rodu *Solanum* byly fúzovány mezofylové protoplasty *Solanum tuberosum s* mezofylovými protoplasty *S. berthaultii a S. verrucosum).* Směs protoplastů v poměru 1:1 byla nakapána na dno Petriho misky (35 mm, 4 kapky, kapka = 100 μl směsi, hustota směsi 10^6 protoplastů na ml). Fúze protoplastů byla uskutečněna pomocí 33 % roztoku polyethylenglykolu (PEG 6000, SERVA) po dobu 15 minut (50μl na každou kapku protoplastové směsi) , poté bylo působení roztoku s PEG ukončeno roztokem STOP podle metody Christey a kol. 1991 (6). Pro následnou kultivaci (*Brassica + Armoracia* nebo *Brassica + Brassica*) byla použita tekutá média dle Pelletier a kol (1983) (7) pro kultivaci *Cucumis* spp. médium LCM1 (3) a pro Solanum spp. SW_{11} (1). Směs protoplastů byla kultivována ve tmě, v termostatu při 25 °C po dobu 72 hodin u fúzi s rodem *Brassica* a 14 dní po fúzi s rodem *Cucumis a Solanum,* po této periodě byly Petriho misky přeneseny do kultivační místnosti s fotoperiodou 16/8 hod den/noc a teplotou 22± 2 °C .

Výsledky a diskuse

Elektrofúze

Protoplasty byly po elektrofúzích byly životné a nepoškozené. Při fúzích byly pozorovány homofúzní i heterofúzní produkty, ale také nefúzované protoplasty. Suspenze protoplastů s fúzním roztokem byla smíchána s kultivačním médiem v poměru 1:1. Protoplasty rodu *Brassica* byly po fúzích kultivovány v tekutém B médiu (7)

a protoplasty rodu *Cucumis* v LCM1 médiu (3). Protoplasty rodu *Solanum* byly smíchány s médiem SW_{11} v dvojnásobné koncentraci všech komponent kromě cukerné složky (již obsažena ve fúzním roztoku) s obsahen 1% agarosy a dále kultivovány ve standardním SW_{11}. Počáteční kultivace po dobu 2 týdnů probíhala ve tmě v termostatu při teplotě 25 °C. Regenerace buněčných stěn a první dělení se objevilo u všech testovaných materiálů v průběhu 6– 8 dnů. Mikrokolonie u rodu *Solanum* se objevily po 15 dnech, mikrokalusy po 22 dnech a první známky organogenze se objevily po 140 dnech. Obdobné výsledky byly získány i při kultivaci nefúzovaných protoplastů odrůd tetraploidního bramboru, které byly testovány z hlediska vhodnosti pro protoplastové kultury. Tekuté medium se snižující se koncentrací osmotika bylo vyměňováno v 7 – 10 denních intervalech do objevení mikrokalusů. Mikrokalusy (*Solanum*) byly dále kultivovány v tekutém médiu C a D podle Sheparda a Tottena (1977) (9) do dosažení regenerace. U nefúzovaných protoplastů bylo dosaženo v některých případech regenerace do stádia celistvé rostliny, po fúzích bylo dosaženo v některých kombinacích prvních známek organogeneze (*S. pinnatisectum* + dihaploid *S. tuberosum*, *S. pinnatisectum* + tetraploid *S. tuberosum*).

Chemické fúze

Po fúzích vznikalo přibližně 10 % heterofúzních produktů, které obsahovaly jak chloroplasty z mezofylových protoplastů tak vakuolární systém z hypokotylových nebo kalusových protoplastů (nebyly počítány homofúzní produkty). Po fúzi byly protoplasty neporušené a životaschopné (obr. 2). Následná kultivace byla v tenké vrstvě tekutého média při hustotě 10^5 protoplastů v 1 ml. Po 24 hodinách protoplasty měnily svůj tvar. Po fúzích s rodem *Brassica* bylo možné pozorovat první buněčné dělení po 2-3 dnech kultivace, mikrokalusy byly získány po 3 týdnech a po 6 týdnech kultivace kalusy (1-2 cm velké). První rostliny byly získány po více než 2 měsících a několikrát pasážovány než byly převedeny do substrátu. U regenerantů byly zaznamenány morfologické odlišnosti *in vitro* i *in vivo*, hybridní rostliny nevykvétaly nebo životnost pylu byla podstatně nižší ve srovnání s donorovými genotypy (8). U mezidruhových fúzí rodu *Cucumis* bylo první buněčné dělení pozorováno 7 dnů po fúzích, druhé dělení po 10-11 dnech a ojediněle mikrokalusy po 6 týdnech, které však nekrotizovaly (nepublikováno). První fúzní experimenty u rodu *Solanum* pomocí PEG vedly k regeneraci buněčných stěn a prvnímu dělení. Experimenty nadále pokračují.

Závěr

Použité parametry elektrického pole byly vyhovující pro všechny použité genotypy, nedocházelo k zásadnímu poklesu životnosti protoplastů a po fúzi bylo možno pozorovat regeneraci buněčných stěn, buněčné dělení a další růst do stádia kalusu a získání regenerantů. O vhodnosti kultivačního postupu svědčí schopnost dosažení regenerace intaktních rostlin prostřednictvím protoplastové kultury.

Chemické fúze pomocí PEG byly úspěšné ve všech kombinacích. Hybridní produkty fúze (mezofyl + hypokotyl, mezofyl + kalus) obsahovaly jak velký počet chloroplastů tak bohatý vakuolární systém, tj. charakter obou partnerů fúze.

Získání hybridních rostlin po elektrofúzích nebo chemických fúzích je závislé na mnoha faktorech - metoda izolace protoplastů, metoda fúze, výběr kultivačního média a podmínek kultivace, ale také na výběru genotypu.

Předpokládaným využitím obou metod je příprava výchozího šlechtitelského materiálu obohaceného o geny rezistence, např. z planých druhů.

Poděkování

Tato práce byla podporována projekty MŠMT České republiky OC 843.20, OC 843.90 a NAZV MZe České republiky QF 4108, QD 1356.

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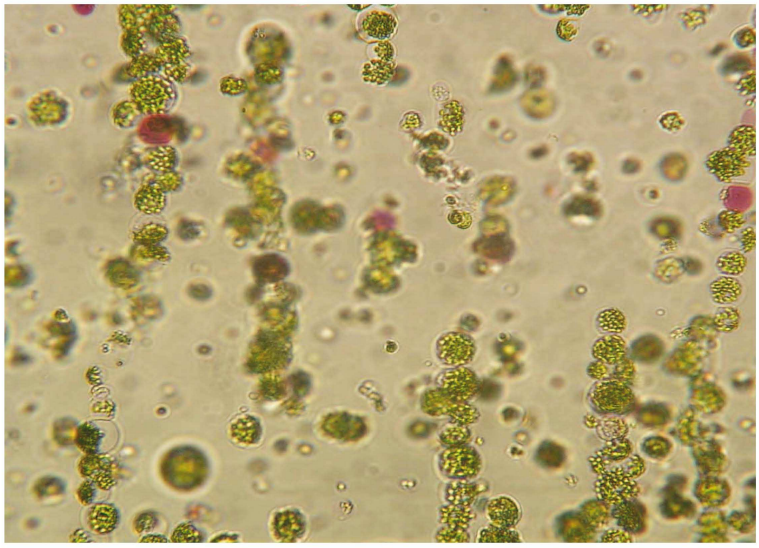
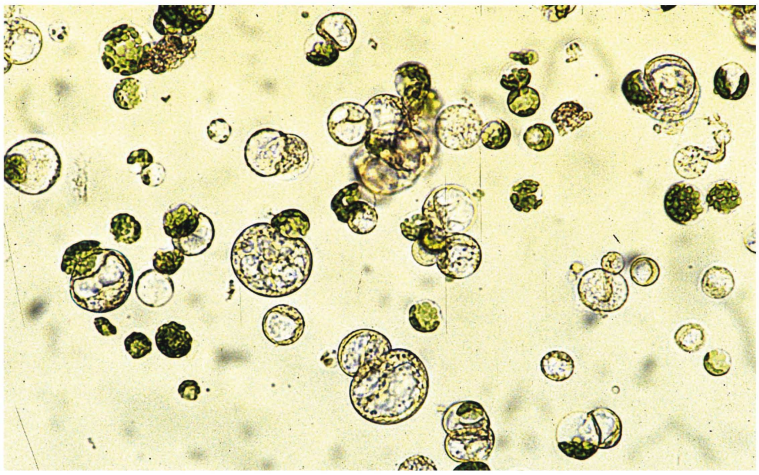


Obr. 1: Řetězení protoplastů v elektrickém poli



Obr. 2: Mezofylové (*Brassica)* a hypokotylové (*Armoracia*) protoplasty 2 hodiny po chemické fúzi

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PROTOPLAST CULTURE AND FUSION BETWEEN *BRASSICA CARINATA* AND *BRASSICA NAPUS*

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Abstract

In this study, attempts were made to select in vitro responsible genotypes and to fuse the isolated protoplasts of Brassica carinata and Brassica napus breeding lines (BC DH Dodolla, BC DH -1, BC DH-6 and BN OP-1, BN-SL-03/04), obtained from our previous experiments. Combination of three different PEG concentrations (20%, 25% or 30%) and two different treatment durations (15 and 20 min.) were tested. Our experiments identified several genotypes (Brassica carinata DH BC-6 and DH BC-1, Brassica napus DH OP-01) with satisfactory regeneration ability of calli from protoplast cultures. Proper combinations of concentration and treatment time of PEG determined protoplast fusion frequency between genotypes used. Although the 30% PEG solution was evaluated to be the best concentration, large amount of multifusants, unwanted in practical applications, was detected especially in Petri dishes with longer PEG treatment. In general, 25% PEG combined with 20 minutes treatment duration produced satisfactory fusion frequency and good rate of viability was obtained as well.

Key words: *Brassica carinata*, *Brassica napus*, protoplast culture, protoplast fusion

INTRODUCTION

The genus *Brassica* includes a wide range of crop species with great economic value worldwide. Therefore, they attracted not only breeders using conventional methods but also those concerned with biotechnological methods. In recent years, major efforts in *Brassica* research have focused on utilization of tissue culture technology for crop improvement. Microspore culture and protoplast culture techniques are most frequently used for the manipulation of foreign gene to broaden genetic diversity. The techniques of protoplast culture and fusion are becoming increasingly useful accessories for breeding programs in various crop plants. The most widespread use of protoplasts is for somatic hybridization experiments either to overcome barriers in sexual crosses or to modify cytoplasmic traits by altering organelle populations (Kumar and Cocking, 1987). Somatic hybridization has been used in *Brassica* improvements programs for the transfer of chloroplast-encoded triazine resistance into cytoplasms carring mitochondria-encoded male sterility (Pelletier et al., 1983) and for the transfer of male sterile cytoplasm from spring to winter rapeseed (Barsby et al., 1987).
Numbers of studies have observed that the *in vitro* tissue culture and the regeneration of plants can lead to alterations in traits conditioned by the cytoplasm (Gengenbach et al., 1981; Kemble et al., 1984). As outlined above, *Brassica* intergeneric, interspecific and intraspecific somatic hybrids and cybrids have been obtained and used for the transfer of agronomically important nuclear and cytoplasmic traits.

Plant regeneration has been increasingly realized via organogenesis and somatic embryogenesis by means of various explants. Protoplast culture improvements have been focused on various factors including genotypes, media additives and effects of enzyme treatment on viability of protoplasts at different time durations.
The aim of our study was to identify *Brassica carinata* and *Brassica napus* genotypes or inbred lines that offer protoplasts with satisfactory regenerative capacity, to optimize current methods for establishing protoplast culture in selected genotypes of *Brassica carinata* and *Brassica napus*, to develop an efficient and reliable protoplast culture protocol for fusion experiments in selected *Brassica carinata* and *Brassica napus* genotypes.

MATERIALS AND METHODS

Plant material

High productive doubled haploid (DH) lines of winter oilseed rape (*Brassica napus* L.) as a source of mesophyll protoplasts BN-OP-01 and BN-SL-03/04 and genotypes of Ethiopian mustard (*Brassica carinata* A. Braun) – DH line BC Dodolla and breeding materials BC-1 and BC-6 – as a source of hypocotyl protoplasts were used for experiments. To obtain sterile plant material for establishing protoplast cultures, seeds of above mentioned genotypes were surface sterilized in 70% ethanol for one minute, followed by immersion in 30% commercial bleach Savo (on the basis of NaClO) for twenty minutes and finally by washing procedure

with sterile distilled water three times. MS medium without growth regulators was used for germination of seedlings. Seeds of genotypes intended for hypocotyl protoplasts were incubated in a thermostat at 25°C in the dark; those for mesophyll protoplasts were kept in a cultivation room and cultivated under controlled environmental conditions (25°C and 16/8h day/night photoperiod and light intensity 260 μmol/m²/s).

Protoplast isolation and fusion

Seven-day old hypocotyls and leaves from one-month-old *in vitro* plants were cut into 1mm segments and treated separately in different Petri dishes with the enzyme solution, containing 0.25% macerozyme R10 (Serva) and 1% cellulase Onozuka R 10 (Serva) in W5 salt solution (Potrykus and Shillito, 1986, Mukhopadhyay et al., 1994). Cut tissue was incubated for 18 hours in a thermostat at 25°C without shaking. Incubated mixtures were filtered separately through a 50 μm nylon mesh and transferred to 10 ml centrifuge tubes. Suspensions were centrifuged at 100 g for 5 minutes. After careful supernatant removal, 4 ml of 20% sucrose solution was mixed with the protoplast suspension and then gently covered with 2 ml W5 salt solution without disturbing the protoplast suspension. Suspensions were again centrifuged at 100g for 5 minutes. Floating protoplasts, forming a ring between two layers of W5 and sucrose solutions, were collected by sterile Pasteur pipette and diluted in the W5 solution (Menczel et al., 1981) and centrifuged at 100 g for 5 minutes.

Protoplasts, intended for fusion experiments were after supernatant removal finally resuspended in 0.1–0.5 ml of M+C medium, suspensions of mesophyll and hypocotyl protoplasts were then mixed in the ratio of 1 : 1 and the density was adjusted to $1–2 \times 10^5$ protoplasts per 1 ml. 3 × 50 μl (3 drops) of the protoplast suspension was added to the 35 mm Petri dish and sedimented for 20 minutes. Polyethylene glycol solution (PEG) was gently added to each drop of sedimented protoplasts and incubated for 15 or 20 minutes. Three different concentrations of PEG 6000 (Molecular weight) were used separately (20%, 25% or 30%) for each set of experiments. The PEG solution was carefully removed and 200 μl of STOP solution was added to each drop. After 20 minutes of incubation the STOP solution was replaced by 0.5 ml of liquid cultivation medium B.

Protoplast cultivation

Protoplasts after isolation, purification and, eventually after fusion experiments were cultured in the liquid medium B (Pelletier et al., 1983), containing 0.25 mg/l 2.4-D, 1 mg/l NAA, 1 mg/l BAP, 2% glucose, pH 5.8 at the density of 2.0×10^4/ml. Petri dishes with protoplast suspension were sealed with Parafilm and incubated in the dark in a thermostat at 25°C. Low osmotic medium C (Pelletier et al., 1983) without 2.4-D was added in amount 0.5 ml per each Petri dish containing medium B. Medium C was added twice every third day. After three weeks of cultivation in the dark, cultures were transferred to a cultivation room and further maintained under controlled environmental conditions (25°C and 16/8h day/night photoperiod and light intensity 260 μmol/m²/s). When microcalli reached the size of 0.5–1.0 mm in diameter, they were transferred to solid induction medium E (Pelletier et al., 1983). The microcalli (calli with typical prolonged cells) formation frequency was calculated by the number of microcalli formed to the total number of protoplasts cultivated.

Plant regeneration

After the growth period of approximately 15 days on the solid E medium calli reached the size of 5–10 mm in diameter. Well-developed calli were transferred to the regeneration medium F (Pelletier et al., 1983) to form shoots. Well-formed shoots were subsequently transferred to MS regeneration medium (Murashige and Skoog, 1962) without growth regulators. After a culture period of 1–2 months on hormone free medium most shoots regenerated roots and were transferred to soil and gradually adapted to a greenhouse environment.

Analysis of data

To prepare the sets of measured values for the analysis of variance (i.e. to follow normal distribution of errors), the percentage data was converted via square-root transformation (Bartlett 1936). Analytic software STATISTICA (StatSoft, Inc., Tulsa, OK, USA) was used for both data analysis and the preparation of graphs.

RESULTS

Regeneration of microcalli

Formation of the cell wall and, subsequently, cell divisions (Figure 6) after 4–7 days of the culture and the regeneration of microcallus structures after four weeks of cultivation (Figure 7) was observed in all genotypes tested and in all biological replications (Table 1). According to the statistical analysis of variance, the effect of a genotype on the regeneration of microcalli was significant, while the influence of individual biological replications and the interaction between genotypes and replication were not relevant (Table 3). The highest frequency of microcalli was obtained in the genotype BC-6 (31.4×10^{-2}%). Significantly less efficient were genotypes BC-1 (22.3×10^{-2}%) and BC Dodolla (18.9×10^{-2}%), followed by BN-OP-01 (13.7×10^{-2}%) and BN-SL-03/04 with 13.4×10^{-2}% of regenerated microcalli per cultivated protoplasts

(Figure 1). Homogeneous groups and contrast values derived from multiple comparisons between means of microcalli regeneration are shown in Table 1.

Regeneration of calli

Induction of callus structures with prolonged cells was detected in all genotypes tested. However, in some biological replications no conversion from microcalli to calli was observed in case of the genotype BN-SL-03/04 (Table 1). These differences were confirmed by means of statistical calculations, where the effects of biological replications (and the genotype, respectively) were evaluated as significant (Table 4). In general, the efficiency of conversion from microcalli to calli was significantly the highest in the genotype BC-6 (23.6%); less efficient were genotypes BC-1 (13.6%) and BN-OP-01 (10.0%). Poor regeneration was observed in case of genotypes BC Dodolla and BN-SL-03/04, where the conversion rates from microcalli to callus structures was only 2.7 and 0.8% (Figure 2). Since there were some differences between biological replications, analyses for individual replications were made as well (Figure 3). Homogeneous groups and contrast values derived from multiple comparisons between means of the callus regeneration are shown in Table 1.

Plant regeneration

In total, sixteen plants with properly developed stems and leaves were regenerated from calli possessing meristematic structures (Figure 8) of genotypes BC-6, BC-1 and BN-OP-1 (Table 1, Figure 9 and 10). Plants with well-developed leaves, regenerated on the solid F medium were transferred to the medium MS without growth regulators. After one week of cultivation on the solid MS medium, regeneration of the root system entered. Plants with properly developed roots were then subsequently multiplied from nodal segments with axilary meristems (Figure 11 and 12). Calli of other genotypes tested were not responsible to regenerate shoots or even meristematic structures and shoot primordia in the callus tissue and turned brown after several subcultivations.

Protoplast fusion

Statistical calculations of the data on the efficiency of the PEG treatment demonstrate its significant impact on the fusion frequency between genotypes used (Table 5). Remarkable differences were detected between individual biological replicates as well (Table 5). In general, the best combinations of 'PEG concentration × time duration' were 25% for 20 minutes and both time duration (15 and 20 minutes) of 30% PEG with fusion frequencies 7.9, 7.8 and 9.1%, respectively. Other combinations were significantly less efficient (Figure 4). More detailed results, i.e. for individual biological replications are shown in Figure 5, homogeneous groups and contrast values based on multiple comparisons between means of the fusion frequency are demonstrated in Table 2. Calli, obtained from fusions between *B. carinata* BC-6 and *B. napus* BN-OP-01 turned yellow and stopped their development (Figure 13).

DISCUSSION

Although the formation of cell divisions and microcallus structures were detected in all genotypes used in our experiments and the progress of the development was similar to the discovery of other researchers (Glimelius, 1984; Hu et al., 1999; Chen et al., 2004, Deep Kaur et al., 2006) there were remarkable differences between particular genotypes not only in the productivity (i.e. in the number of microcalluses per cultivated protoplasts) but also in the morphological and physiological characteristics of obtained microcalli. While the genotype BC-6 proved to be the most productive (31.4×10^{2}% of regenerated microcalli per cultivated protoplasts), genotypes BN-OP-01 and BN-SL-03/04 demonstrated rather low level of regeneration ability. In addition, above mentioned, low productive genotypes often formed microcalli that gradually turned brown at early stages of development. Such structures may exude various substances, which can negatively affect the development of other, normally dividing microcalli (Chen et al., 2004). Strong impact of the genotype on the initialisation of cell divisions and further development of dedifferentiated tissue in the protoplast culture was presented by various authors like Hu et al., 1999 in *B. napus* and *B. juncea*; Chen et al., 2004 in *B. oleracea*; Narasimhulu et al., 1992 in *B. carinata.*

Successful conversion of microcalli to calli was observed in all genotypes used; however, outstanding differences in regeneration ability were detected not only between individual genotypes but also between some biological replicates within particular genotypes. The relevance of both factors was later confirmed via statistical analyses of measured data. Moreover, in some biological and technical replicates no regeneration of calli, characterised by formation of prolonged cells, was detected, although such genotypes produced satisfactory amount of calluses in other replications. For example, viable calli were regenerated only from one biological replication (B) of the genotype BN-SL-03/04. The differences in the efficiency of the callus formation between genotypes have been reported by many authors, for instance Hu et al. (1999); Deep Kaur et al. (2006). Significant differences between some biological (i.e. successive) replications of the same genotype can be explained by the heterogeneity of the biological material used for establishing of protoplast cultures and was observed also in the experiments of Chen et al. (2004).

Plants with properly developed leaves and stems were derived from bright green calluses with dark green meristematic structures inside callus tissue. No shoots were regenerated from white and yellow calluses without meristematic zones. In total, only sixteen calluses of genotypes BC-1, BC-6 and BN-OP-01 regenerated shoots; several previously mentioned calli produced more than one shoot. All regenerated plants were characterized by certain level of hyperhydricity; such type of plants was described also by Jourdan and Earle (1989) in the protoplast culture of four *Brassica* species, Hu et al., 1999 in *Brassica napus and B. juncea*. This undesirable physiological state was eliminated by repeated subcultivation on MS medium without growth regulators.

It is evident, that only the proper combination of PEG 6000 concentrations and time durations can provide satisfactory fusion frequency. From results can be concluded that all the 30 % PEG solutions provided good results (about 8% of fusants) together with 25% PEG solution with the period of 20 minutes. The efficiency of these combinations is comparable with the results, obtained by Gurel et al. (2002). Even higher frequency of fusants was observed by Beranek et al. (2006) on protoplast fusions between *B. carinata* and *B. rapa*, where the 30% PEG 6000 MW treatment for 10 minutes achieved about 25% of fusants. Both 20% PEG concentrations and shorter period of 25% concentration showed rather low efficiency. However, both combinations with 30% PEG solution increased the number of multifusants, unfavourable for practical applications. Analogous to the production of calli, significant differences between biological replicates in fusion frequency were detected as well. These circumstances might be explained by the heterogeneity of fused material during experiments even if all measurable environmental conditions were maintained. Protoplasts of fused cultures regenerated cell walls and first divisions were observed after 4th day of the culture. Small calli, approx. 1-2 mm in diameter turned brown-yellow and did not develop desired callus structures. Similar results were defined by Beranek et al. (2007) in *Brassica carinata* and *Brassica rapa* genotypes.

CONCLUSION

Regeneration of microcalli and calli was achieved in all examined genotypes; strong effect of the genotype on the regeneration of both types of dedifferentiated tissue was confirmed by means of statistical analysis. *Brassica carinata* BC-6 and BC-1 were proved the best genotypes in terms of the formation of microcalli and calli from isolated protoplasts. On the contrary, not satisfactory level of regeneration ability was reported in genotypes *B. carinata* BC Dodolla and *B. napus* BN-SL-03/04. However, significant differences were detected also between biological replicates within some genotypes tested; this phenomenon might be caused by certain level of the heterogeneity of biological material used for successive replications.

Whole plants with well-developed roots were derived from three genotypes; only calli with dark green meristematic structures were able to regenerate shoot primordia and subsequently shoots, however, hyperhydrated breakable stems and leaves were observed in all achieved regenerants. Consequently, repeated subcultivations on the solid MS medium were necessary to obtain vigorous plants.

Proper combinations of PEG concentrations and treatment time determined the fusion frequency between genotypes used. Although the 30% PEG solution was evaluated to be the best concentration, large amount of multifusants, unwanted in practical applications, was detected especially in Petri dishes with longer PEG treatment.

Our experiments identified several genotypes with satisfactory regeneration ability of calli from protoplast cultures. However, poor regeneration of whole plants and their physiological state (i.e. hyperhydricity) extended the regeneration of vigorous plants and thus complicated transferring such plants to non-sterile conditions.

Although callus-like aggregates, achieved in fused cultures, did not formed calli with the ability to undergo further regeneration, optimal concentrations and time durations of the fusion agent polyethylene glycol were evaluated for *B. carinata* BC-1 and *B. napus* BN-OP-01. These observations could help in further research not only on fusion experiments with closely related species of genus *Brassica.*

Tab. 1: Microcalli, calli and plant regeneration in the protoplast culture of selected genotypes of genus *Brassica*

Replication (+)		(A)				(B)				(C)			
Genotype	Petri Dish No. (++)	No. of microcalli (*)	No. of calli (*)	Regeneration of microcalli (**) [x 10 $^{-2}$ %]	Regeneration of calli (***) [%]	No. of plants per Petri Dish (*)	No. of microcalli (*)	No. of calli (*)	Regeneration of microcalli (**) [x 10 $^{-2}$ %]	Regeneration of calli (***)	No. of plants per Petri Dish [%] (*)	No. of microcalli (*)	No. of calli (*)	Regeneration of microcalli (**) [x 10 $^{-2}$ %]	Regeneration of calli (***) [%]	No. of plants per Petri Dish (*)
BC Dodolla	1	238.5	0.0	18.3	0.0	0	184.6	15.4	14.2	8.3	0	269.3	7.7	20.7	2.9	0
	2	207.7	0.0	16.0	0.0	0	253.9	7.7	19.5	3.0	0	176.9	0.0	13.6	0.0	0
	3	261.6	7.7	20.1	2.9	0	230.8	7.7	17.8	3.3	0	392.3	15.4	30.2	3.9	0
Mean		235.9	2.6	def 18.1	EF 1.0		223.1	10.3	def 17.2	CD 4.9		279.5	7.7	cde 21.5	DE 2.3	
BC-6	1	407.7	107.7	31.4	26.4	2	469.3	138.5	36.1	29.5	1	538.5	115.4	41.4	21.4	1
	2	323.1	69.2	24.9	21.4	0	338.5	84.6	26.0	25.0	0	438.5	69.2	33.7	15.8	0
	3	338.5	92.3	26.0	27.3	0	430.8	100.0	33.1	23.2	2	384.7	84.6	29.6	22.0	0
Mean		356.4	89.8	abc 27.4	A 25.0		412.9	107.7	ab 31.8	A 25.9		453.9	89.8	a 34.9	AB 19.7	
BC-1	1	307.7	46.2	23.7	15.0	2	284.6	23.1	21.9	8.1	0	269.3	30.8	20.7	11.4	1
	2	284.6	30.8	21.9	10.8	2	338.5	61.5	26.0	18.2	0	415.4	69.2	32.0	16.7	0
	3	215.4	23.1	16.6	10.7	0	238.5	30.8	18.3	12.9	0	253.9	46.2	19.5	18.2	2
Mean		269.3	33.3	cde 20.7	B 12.2		287.2	38.5	cde 22.1	B 13.1		312.8	48.7	bcd 24.1	AB 15.4	
BN-OP-01	1	253.9	23.1	19.5	9.1	0	146.2	23.1	11.2	15.8	0	192.3	15.4	14.8	8.0	1
	2	323.1	38.5	24.9	11.9	2	153.9	23.1	11.8	15.0	0	92.3	0.0	7.1	0.0	0
	3	200.0	23.1	15.4	11.5	0	76.9	7.7	5.9	10.0	0	169.2	15.4	13.0	9.1	0
Mean		259.0	28.2	cde 19.9	BC 10.8		125.7	18.0	g 9.7	D 13.6		151.3	10.3	fg 11.6	D 5.7	
BN-SL-03/04	1	161.6	0.0	12.4	0.0	0	200.0	7.7	15.4	3.8	0	115.4	0.0	8.9	0.0	0
	2	92.3	0.0	7.1	0.0	0	176.9	0.0	13.6	0.0	0	200.0	0.0	15.4	0.0	0
	3	246.2	0.0	18.9	0.0	0	238.5	7.7	18.3	3.2	0	138.5	0.0	10.7	0.0	0
Mean		166.7	0.0	fg 12.8	F 0.0		205.1	5.1	efg 15.8	DE 2.4		151.3	0.0	fg 11.6	F 0.0	

(*), per 35 mm Petri Dish; (**), per No. of cultivated protoplasts; (***), per No. of obtained microcalli, (+), Biological replicates; (++), Technical replicates
Letters in rows and columns indicate homogeneous groups derived from multiple comparisons between means of microcalli (a – f) and calli regeneration (A – F) (LSD; $P = 0.05$)

Tab. 2: Fusion frequency between *B. carinata* BC-6 and *B. napus* BN-OP-01 genotypes obtained from different treatments with PEG solution

Replication (+)		(A)			(B)			(C)		
Concentration of PEG solution	Spot No. (+)	No. of protoplasts (*)	No. of fusants (*)	Fusion frequency [%]	No. of protoplasts (*)	No. of fusants (*)	Fusion frequency [%]	No. of protoplasts (*)	No. of fusants (*)	Fusion frequency [%]
PEG 20% 15 minutes	1	31.2	1.2	3.8	36.6	0.2	0.5	69.8	1.6	2.3
	2	56.8	1.0	1.8	87.2	1.0	1.1	75.0	4.0	5.3
	3	47.6	0.2	0.4	49.8	0.4	0.8	65.4	2.8	4.3
	Mean	45.2	0.8	fg 2.0	57.9	0.5	g 0.8	70.1	2.8	def 4.0
PEG 20% 20 minutes	1	77.0	1.0	1.3	56.6	1.4	2.5	83.2	2.0	2.4
	2	65.8	0.8	1.2	39.0	0.4	1.0	49.0	1.4	2.9
	3	59.2	1.0	1.7	77.6	0.0	0.0	63.0	4.0	6.3
	Mean	67.3	0.9	fg 1.4	57.7	0.6	g 1.2	65.1	2.5	def 3.9
PEG 25% 15 minutes	1	80.8	2.2	2.7	62.6	0.0	0.0	88.2	2.8	3.2
	2	44.2	1.0	2.3	75.2	1.6	2.1	76.6	4.6	6.0
	3	95.4	3.6	3.8	93.2	2.2	2.4	84.0	5.0	6.0
	Mean	73.5	2.3	efg 2.9	77.0	1.3	g 1.5	82.9	4.1	cde
PEG 25% 20 minutes	1	74.4	4.0	5.4	69.0	2.0	2.9	105.2	6.8	6.5
	2	100.6	5.6	5.6	51.4	3.8	7.4	71.8	9.0	12.5
	3	62.2	8.2	13.2	85.0	8.0	9.4	91.8	7.6	8.3
	Mean	79.1	5.9	abcd	68.5	4.6	bcde	89.6	7.8	abc
PEG 30% 15 minutes	1	37.6	3.6	9.6	45.8	1.6	3.5	67.2	7.2	10.7
	2	87.2	5.2	6.0	38.6	3.6	9.3	59.8	4.8	8.0
	3	99.0	5.0	5.1	69.2	5.2	7.5	81.0	8.2	10.1
	Mean	74.6	4.6	bcd	51.2	3.5	bcde	69.3	6.7	ab 9.6
PEG 30% 20 minutes	1	71.8	6.6	9.2	69.0	5.2	7.5	81.2	14.0	17.2
	2	60.6	4.2	6.9	64.4	4.0	6.2	95.0	11.4	12.0
	3	89.0	7.4	8.3	96.6	3.8	3.9	74.8	7.8	10.4
	Mean	73.8	6.1	abc	76.7	4.3	bcde	83.7	11.1	a 13.2

(*) per 1 mm^2 of fused spots
(+) Biological replicates; (++) Technical replicates
Letters a–g in rows and columns indicate homogeneous groups derived from multiple comparisons between means of fusion frequency (LSD; $P = 0.05$)

Tab. 3: Test of significance (P) of individual sources of variability and their interactions for the regeneration of microcalli

Effect	SS	DoF	MS	F	P
Genotype	0.241811	4	0.060453	20.684	0.000000*
Replication	0.001300	2	0.000650	0.222	0.801899
Genotype x Replication	0.046789	8	0.005849	2.001	0.080878
Error	0.087680	30	0.002923		

Tab. 4: Test of significance (P) of individual sources of variability and their interactions for the regeneration of calli

Effect	SS	DoF	MS	F	P
Genotype	113.8755	4	28.4689	53.2550	0.000000*
Replication	5.9839	2	2.9919	5.5969	0.008597*
Genotype x Replication	6.9016	8	0.8627	1.6138	0.162608
Error	16.0373	30	0.5346		

Tab. 5: Test of significance (*P*) of individual sources of variability and their interactions for fusion frequency between *B. carinata* BC-6 and *B. napus* BN-OP-01

Effect	SS	DoF	MS	F	P
Replication	7.9239	2	3.9620	14.6779	0.000022*
PEG solution	26.0091	5	5.2018	19.2711	0.000000*
Replication x PEG solution	0.9852	10	0.0985	0.3650	0.953861
Error	9.7174	36	0.2699		

Two-way ANOVA; significant effects (*P*-value ≤ α-level = 0.05) are marked with an asterisk (*)
SS – Sum of Squares, DoF – Degrees of Freedom, MS – Mean Square, F – F-Statistic, *P* – Significance (*P*-value)

Figure 1: The impact of individual genotypes on the regeneration of microcalli; pooled data for three successive biological and three technical replicates

Bars represent individual 95% confidence intervals
Letters a-c designate homogeneous groups (LSD; *P* = 0.05)

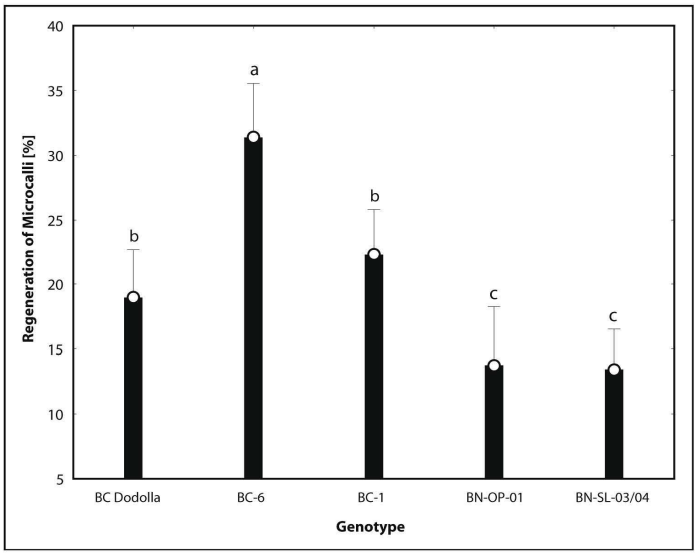

Figure 2: The impact of individual genotypes on the regeneration of calli; pooled data for three successive biological and three technical replicates

Bars represent individual 95% confidence intervals
Letters a–d designate homogeneous groups (LSD; *P* = 0.05)

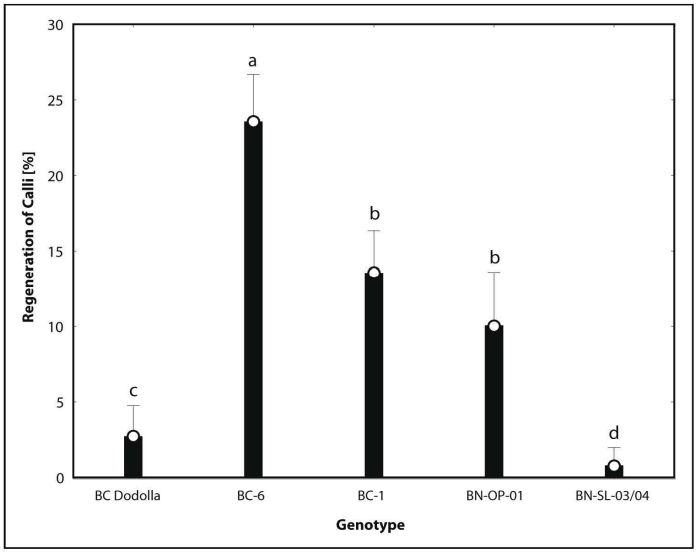

Figure 3: The impact of particular genotypes on the regeneration of calli within individual biological replicates (%); pooled data for three technical replicates

Bars represent individual 95% confidence intervals
Letters a–c, A–C, and α–γ designate homogeneous groups (LSD; $P = 0.05$)

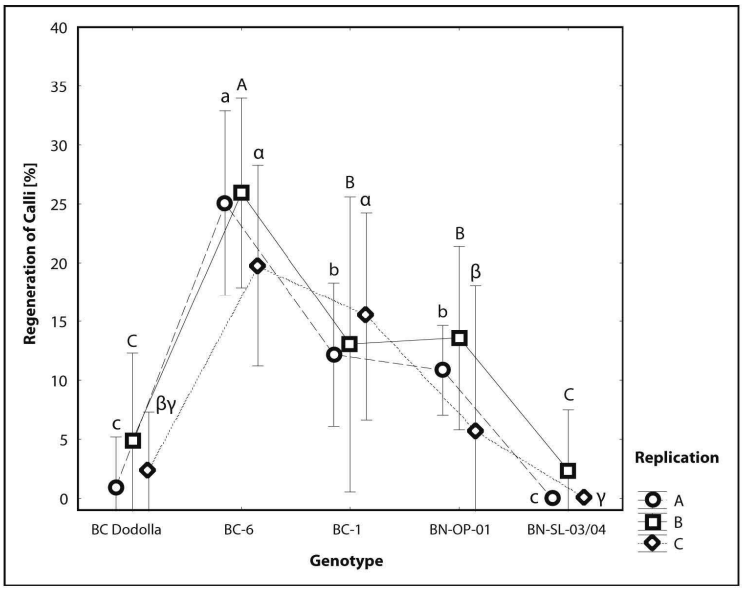

Figure 4: The impact of different PEG treatments on the fusion frequency between *B. carinata* BC-6 and *B. napus* BN-OP-01 genotypes (%); pooled data for three successive biological and three technical replicates

Bars represent individual 95% confidence intervals
Letters a and b designate homogeneous groups (LSD; $P = 0.05$)

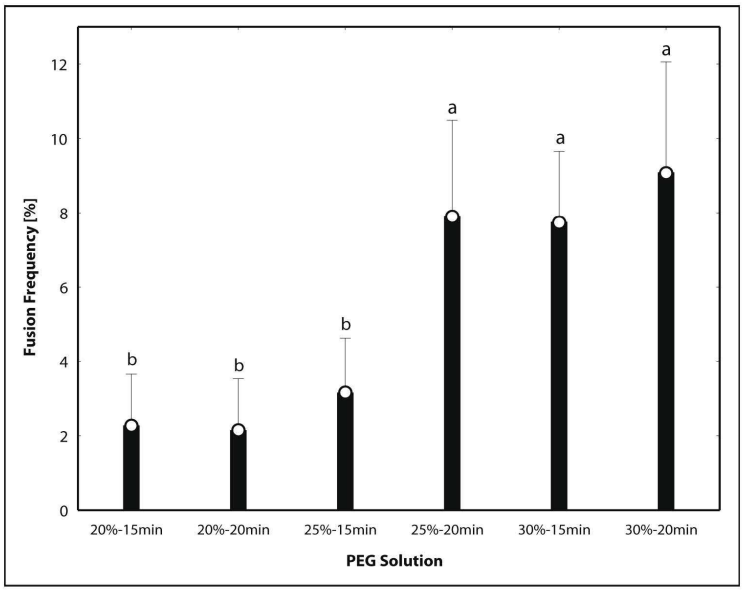

Figure 5: The impact of different PEG treatments on the fusion frequency (%) between *B. carinata* BC-6 and *B. napus* BN-OP-01 genotypes, within individual biological replicates; pooled data for three technical replicates

Bars represent individual 95% confidence intervals
Letters a–b, A–B, and α–γ designate homogeneous groups (LSD; $P = 0.05$)

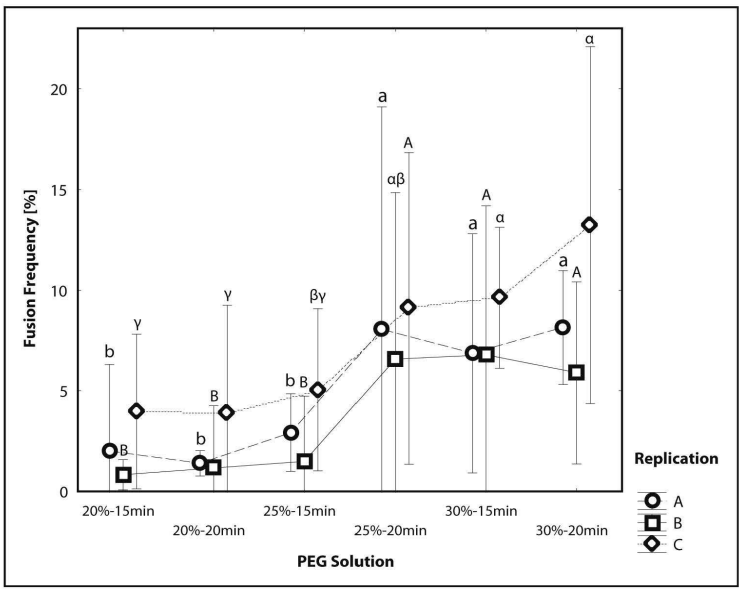

Figure 6: Cell divisions in the protoplast culture of the genotype BC-6 (Liquid C medium)

Bar = 50 micrometers

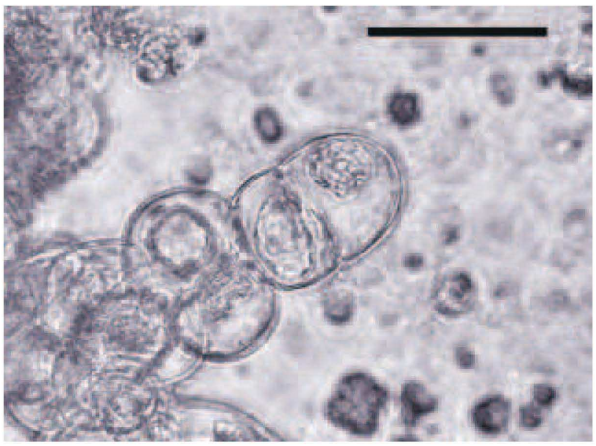

Figure 7: Formation of the microcallus in the protoplast culture of the genotype BC-6 (Liquid C medium)

Bar = 50 micrometers

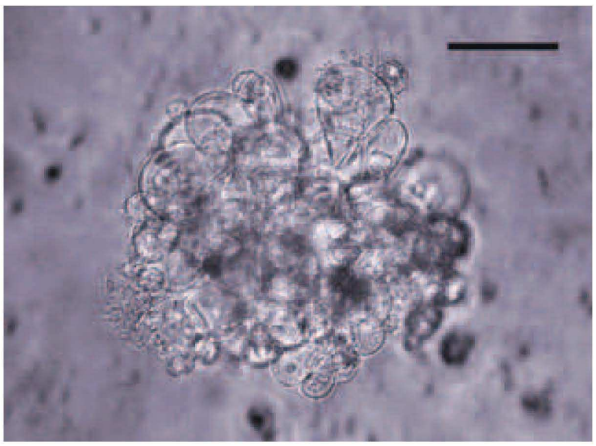

Figure 8: Protoplast-derived with meristem-like structures of the genotype BC-6 (Solid F medium)

Bar 5 millimetres

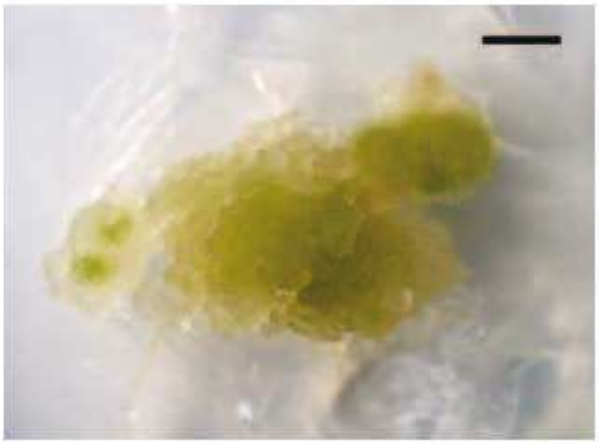

Figure 9: Plant regeneration from protoplast-derived callus tissue of the genotype BN-OP-01 (Solid F medium)

Petridish diameter 90 millimetres

Figure 10: Plant regeneration from protoplast-derived callus tissue of the genotype BC-1 (Solid F medium)

Petridish diameter 90 millimetres

Figure 11: Whole plants of the genotype BN-OP-01 on the solid rooting medium MS

Flask height 100 millimetres

Figure 12: Whole plants of *Brassica carinata* genotypes BC-1 and BC-6 on the solid rooting medium MS

Flask height 100 millimetres

Figure 13: Small calli, regenerated from fused culture of *B. carinata* BC-6 and *B. napus* BN-OP-01 genotypes (Liquid C medium)

Petridish diameter 35 millimeters

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FÚZE PROTOPLASTŮ U HOSPODÁŘSKY VÝZNAMNÝCH BRUKVOVITÝCH PLODIN

Protoplast fusion in agriculturally important cruciferous crops

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Abstrakt
Cílem experimentů bylo vybrat v protoplastových kulturách dobře regenerující dihaploidní linie ozimé řepky a hořčice habešské a stanovit optimální parametry chemických fúzí protoplastů u vybraných genotypů. Byly testovány tři koncentrace polyetylén glykolu (PEG 6000) ve dvou variantách doby ošetření. Byly identifikovány tři genotypy s uspokojivou regenerační schopností. Nejvyšší frekvence fúzantů v kombinaci s dobrou životností produktů fúze byla u sledovaných genotypů zjištěna při použití 25% PEG po dobu 20 minut.
Klíčová slova*:* *Brassica carinata*, *Brassica napus*, protoplastové kultury, fúze protoplastů

Abstract
The aim of presented experiments was to select *in vitro* responsible genotypes and to fuse the isolated protoplasts of *Brassica carinata* and *Brassica napus* breeding lines. Combination of three different PEG concentration (20%, 25% or 30%) and two different treatment durations (15 and 20 min.) were tested. Our experiments identified several genotypes (*Brassica carinata* DH BC-6 and DH BC-1, *Brassica napus* DH OP-01) with satisfactory regeneration ability of calli from protoplast cultures. In general, 25% PEG combined with 20 minutes treatment duration produced good fusion frequency and good rate of viability was obtained as well.
Keywords*: Brassica carinata*, *Brassica napus*, protoplast culture, protoplast fusion

Úvod
Fúze protoplastů představuje unikátní nástroj, umožňující přenos znaků a vlastností, podmíněných geny jádra nebo cytoplazmy. Některé krajové odrůdy rodu *Brassica* se vyznačují z agronomického hlediska významnými vlastnostmi (například rezistencí k významným chorobám), které se mohou uplatnit u kulturních druhů po jejich přenesení pomocí fúzí protoplastů. Somatická hybridizace byla u brukvovitých mj. použita i pro přenos chloroplasty podmíněné rezistence k triazinu (Pelletier *et al*. 1983) nebo cytoplazmatické samčí sterility z jarní do ozimé řepky (Barsby *et al*. 1987). Perspektivní plodinou pro tyto manipulace může být např. i hořčice habešská (*B. carinata*), vyznačující se rezistencí k suchu a širokému spektru chorob a škůdců (Malik 1990). Pro úspěšnou realizaci somatických hybridizací je ale nezbytné získat potřebné množství životných protoplastů a optimalizovat protokol pro jejich fúzi a následnou regeneraci (Jha, Ghosh 2005).
Cílem prezentované práce bylo identifikovat z krajových odrůd odvozené dihaploidní genotypy hořčice habešské a ozimé řepky (*B. napus*) s dobrou regenerační schopností v protoplastových kulturách a optimalizovat vybrané parametry chemických fúzí u těchto genotypů.

Materiál a metody

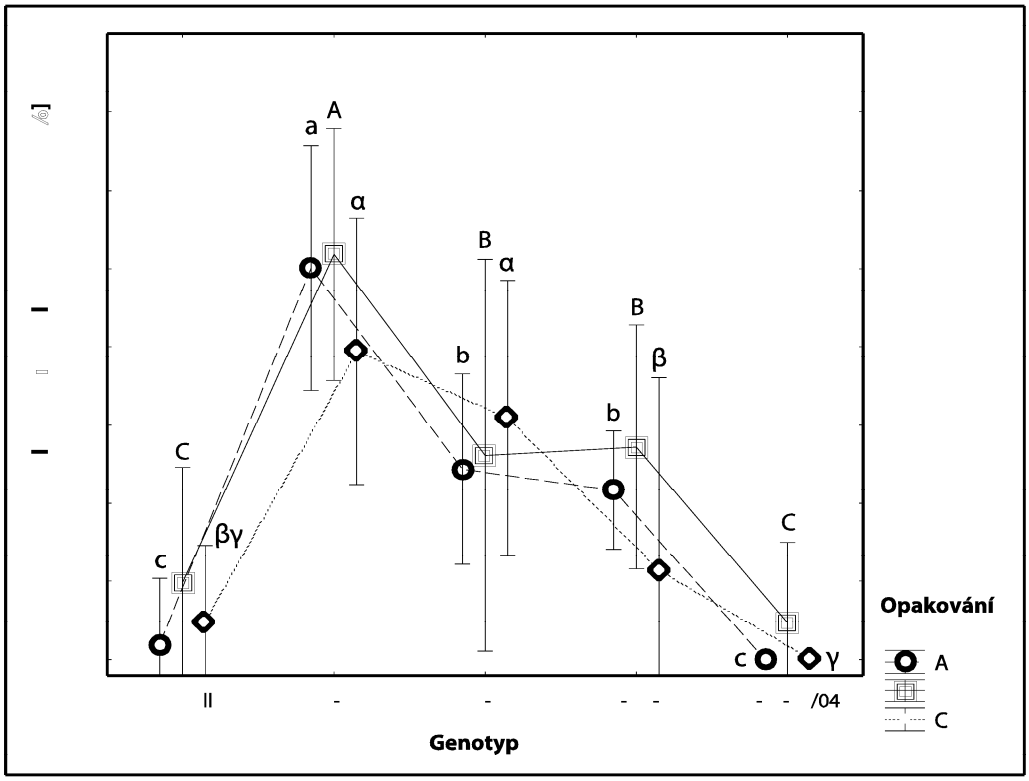
Dihaploidní (DH) linie ozimé řepky BN-OP-01 a BN-SL-03/04 byly použity jako zdroj mezofylových protoplastů; DH linie *B. carinata* Dodolla, BC-1 a BC-6 byly zdrojem hypokotylových protoplastů. Příprava donorových rostlin, izolace, purifikace a příprava protoplastů k fúzím byla provedena dle Beránka *et al.* (2007).
Byly testovány tři různé koncentrace polyetylén glykolu (PEG) 6000 MW (20, 25 a 30%) po dobu 15 nebo 20 minut. Po uplynutí stanovené doby byl roztok PEG odstraněn a přidán roztok STOP. Po 20 minutách byl tento odstraněn a dodáno kultivační médium B (Pelletier *et al*. 1983). Další kultivace probíhala dle Kaur *et al*. (2006) a Beránka *et al.* (2007).

Tabulka 1. Frekvence fúzí mezi *B. carinata* BC-6 a *B. napus* BN-OP-01 v závislosti na různých kombinacích koncetrace PEG a doby působení.

Opakování		A			B			C		
Koncentrace PEG roztoku	terčíkuČíslo	Počet protoplastů (*)	Počet fúzantů (*)	Frekvence fúzí [%]	Počet protoplastů (*)	Počet fúzantů (*)	Frekvence fúzí [%]	Počet protoplastů (*)	Počet fúzantů (*)	Frekvence fúzí [%]
PEG 20 %	1	31,2	1,2	3,8	36,6	0,2	0,5	69,8	1,6	2,3
15 minut	2	56,8	1,0	1,8	87,2	1,0	1,1	75,0	4,0	5,3
	3	47,6	0,2	0,4	49,8	0,4	0,8	65,4	2,8	4,3
	Průměr	**45,2**	**0,8**	**fg 2,0**	**57,9**	**0,5**	**g 0,8**	**70,1**	**2,8**	**def 4,0**
PEG 20 %	1	77,0	1,0	1,3	56,6	1,4	2,5	83,2	2,0	2,4
20 minut	2	65,8	0,8	1,2	39,0	0,4	1,0	49,0	1,4	2,9
	3	59,2	1,0	1,7	77,6	0,0	0,0	63,0	4,0	6,3
	Průměr	**67,3**	**0,9**	**fg 1,4**	**57,7**	**0,6**	**g 1,2**	**65,1**	**2,5**	**def 3,9**
PEG 25 %	1	80,8	2,2	2,7	62,6	0,0	0,0	88,2	2,8	3,2
15 minut	2	44,2	1,0	2,3	75,2	1,6	2,1	76,6	4,6	6,0
	3	95,4	3,6	3,8	93,2	2,2	2,4	84,0	5,0	6,0
	Průměr	**73,5**	**2,3**	**efg 2,9**	**77,0**	**1,3**	**g 1,5**	**82,9**	**4,1**	**cde 5,0**
PEG 25 %	1	74,4	4,0	5,4	69,0	2,0	2,9	105,2	6,8	6,5
20 minut	2	100,6	5,6	5,6	51,4	3,8	7,4	71,8	9,0	12,5
	3	62,2	8,2	13,2	85,0	8,0	9,4	91,8	7,6	8,3
	Průměr	**79,1**	**5,9**	**abcd 8,0**	**68,5**	**4,6**	**bcde 6,6**	**89,6**	**7,8**	**abc 9,1**
PEG 30 %	1	37,6	3,6	9,6	45,8	1,6	3,5	67,2	7,2	10,7
15 minut	2	87,2	5,2	6,0	38,6	3,6	9,3	59,8	4,8	8,0
	3	99,0	5,0	5,1	69,2	5,2	7,5	81,0	8,2	10,1
	Průměr	**74,6**	**4,6**	**bcd 6,9**	**51,2**	**3,5**	**bcde 6,8**	**69,3**	**6,7**	**ab 9,6**
PEG 30%	1	71,8	6,6	9,2	69,0	5,2	7,5	81,2	14,0	17,2
20 minut	2	60,6	4,2	6,9	64,4	4,0	6,2	95,0	11,4	12,0
	3	89,0	7,4	8,3	96,6	3,8	3,9	74,8	7,8	10,4
	Průměr	**73,8**	**6,1**	**abc 8,1**	**76,7**	**4,3**	**bcde 5,9**	**83,7**	**11,1**	**a 13,2**

(*)- na 1mm^2 terčíku
Písmena a – g v řádcích i sloupcích reprezentují homogenní podskupiny, odvozené z mnohonásobných porovnávání mezi průměry frekvencí fúzí (LSD; P = 0,05)

Graf 1. Vliv jednotlivých genotypů na regeneraci kalusů z mikrokalusových struktur (v %) v rámci individuálních biologických opakování (A, B a C). Data jsou průměrem ze tří technických opakování.



Úsečky reprezentují 95% intervaly spolehlivosti
Písmena a-c, A-C, a α-γ vyjadřují homogenní podskupiny (LSD; *P* = 0,05)

Výsledky a diskuse

I když regenerace kalusového pletiva byla pozorována u všech testovaných genotypů, u některých opakování nebylo dosaženo tvorby kalusu (Graf 1). Toto je ve shodě s výsledky dalších autorů (Hu *et al*. 1999, Chen *et al*. 2004, Kaur *et al*. 2006). Nejvyšší frekvence regenerace kalusů z mikrokalusových struktur bylo dosaženo u genotypu BC-6 (23,6 %); méně úspěšné byly pak genotypy BC-1 (13,6 %) a BN-OP-01 (10,0 %). Nízká regenerace byla pozorována u genotypů BC Dodolla a BN-SL-03/04, kde úroveň konverze byla pouze 2,7, respektive 0,8 %. Významný vliv genotypu na inicializaci dělení buněk a další regeneraci nediferencovaného pletiva byl publikován také dalšími autory (Hu *et al.* (1999) u *Brassica napus*, Chen *et al.* (2004) u *B. oleracea*, Narasimhulu *et al*. (1992) u *Brassica carinata* a Hu *et al.* (1999) u *B. juncea*).

Byl zjištěn významný vliv jednotlivých koncentrací PEG a dob působení na frekvenci fúzí u sledovaných genotypů (Tabulka 1). Významné rozdíly byly zjištěny i mezi opakováními. Nejlepšími kombinacemi ‘PEG koncentrace × doba působení’ byly 25% PEG po 20 minut a 30% PEG při obou dobách působení (15 a 20 minut). Obě 20 % PEG koncentrace a kratší doba působení u 25 % koncentrace přinesla horší výsledky (Graf 1). Je evidentní, že pouze optimální kombinace mezi koncentrací PEG a doby působení zajistí uspokojivou frekvenci fúzí. Podobné frekvence fúzí získal i Gurel *et al.* (2002). Vyšší frekvence pozoroval Beránek *et al.* (2006) u fúzí mezi *B. carinata* a *B. rapa*, kdy v kombinaci 30% PEG 6000 MW po dobu

10 minut bylo získáno okolo 25 % fúzantů. Na druhou stranu, obě úspěšné kombinace s 30% PEG zvýšily nežádoucí výskyt multifúzantů.

Závěr

V našich experimentech bylo identifikováno několik genotypů s dobrou regenerační schopností. Optimální, experimentálně zjištěná kombinace mezi koncentrací PEG a doby působení zajistila uspokojivou frekvenci fúzí. Tato zjištění mohou pomoci v dalších experimentech nejen u blízce příbuzných druhů rodu *Brassica*.

Řešení uvedené problematiky bylo podpořeno MZe ČR, projekt č. 0002700602.

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Electrofusion of Protoplasts in Selected Vegetables of *Brassica*, *Cucumis* and *Solanum* Genera

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Keywords: electrofusion, protoplast, somatic hybridization, *Brassica*, *Cucumis*, *Solanum*

Abstract

The aim of this study is using protoplast electrofusion for somatic hybridization, especially for increasing of genetic variability in selected important vegetable species of *Brassica*, *Cucumis* and *Solanum* genus and possibility of including this method into breeding approaches to obtain new breeding materials. Mesophyll protoplasts were isolated from in vitro cultured plants of *Brassica olearacea* (*B. oleracea* var. *capitata*, *B. oleracea* var. *botrytis*), *Cucumis* spp. (*C. metuliferus*, *C. melo*) and *Solanum tuberosum* dihaploid plants and wild *Solanum* species (*S. pinnatisectum*, *S. bulbocastanum*). Hypocotyl protoplasts were isolated from etiolated seedlings of *Brassica oleracea* (cabbage, cauliflower) and callus protoplasts of *Cucumis melo*. Enzymatic mixture of 1% or 2% Cellulase Onozuka R-10 and 0.25% or 1% Macerozyme R-10 in rinsing solution Pgly/W5 (2,1) was used for isolation of mesophyll and hypocotyl protoplasts or callus protoplasts. Leaves, hypocotyls and/or calli were segmented and put into a thermostat at 25°C for 16-18 hours. Protoplasts were isolated using a conventional method (filtration, centrifugation and purification with 20% sucrose gradient). Purified protoplasts were rinsed with pre-fusion solution (0.4 M manitol and 1 mM $CaCl_2$) and fusion solution (0.4 M manitol), after centrifugation their required density was obtained by dilution with fusion solution. Viability of isolated protoplasts used for electrofusions was more than 80% (determined using FDA). Fusing partner components were mixed in the ratio of 1:1. There were fused protoplasts of *Brassica* genus: mesophyll and hypocotyl, *Cucumis* genus: mesophyll and callus, *Solanum* genus: mesophyll and mesophyll. Protoplast fusion was performed using apparatus ECM 2001 and in electroporation chamber for 20-30 µl with the 0.5 mm electrode distance. Following parameters were used for fusions: for protoplast alignment - 5V AC and 1 MHz frequency with individual duration of action, for fusion - 1 pulse 10V DC of length 80 µs (3). Following electrofusions protoplasts were viable and undamaged. Homofusion and heterofusion products and also unfused protoplasts could be observed. Subsequent culturing was done in medium LCM1 (2) or SW11 (1) in a thermostat at 25°C for 2 weeks in the dark. Regeneration of cell walls was recorded and first division was found in 6-8 days of culture, in *Solanum* genus microcolonies were found in 15 days. The experiments have not been finished yet.

INTRODUCTION

The aim of this study is using protoplast electrofusion for somatic hybridization, especially for increasing of genetic variability in selected important vegetable species of *Brassica, Cucumis* and *Solanum* genera and possibility of including this method into breeding approaches to obtain new breeding materials.

In Cucurbitaceae, production of somatic hybrids by protoplast fusion has been

tried by several researchers, but reliable procedures for obtaining fertile hybrids remained illusive. Most researchers have tried to produce interspecific cybrids involving *Cucumis melo*. There are only two reports dealing with crossing of *Cucumis sativus* with *Cucumis metuliferus*. In those experiments, the regeneration of fused protoplasts stopped at the minicallus stage (Gajdová et al., 2004). In most cases, no regeneration was achieved, or regeneration of normal plants was not observed. The most difficult step was the induction of shoots on the callus and their development into whole plants.

Some wild potato species have been successfuly used in protoplast fusions with cultured potato *S. tuberosum* (Thieme et al., 1997). Somatic hybrids of *B. oleracea* var. *botrytis* and *capitata* with representative of Brassicaceae have been obtained (Navrátilová, 2004).

MATERIAL AND METHODS

Plant Material

Plants of *Brassica oleracea* var. *botrytis* and *capitata, Cucumis metuliferus* and *C. melo* were cultivated in vitro on MS medium. *S. tuberosum* dihaploid plants and diploid wild species of the genus *Solanum* were cultivated in vitro on SH (Schenk and Hildebrandt, 1972) medium with 3 mg/L $AgNO_3$ and 1,5 mg/L Alar 85.

Sources of mesophyl protoplasts were plants of *Brassica olearacea* (*B. oleracea* var. *capitata*, *B. oleracea* var. *botrytis*), *Cucumis* spp. (*C. metuliferus*, *C. melo*) and *Solanum tuberosum* dihaploid plants and wild *Solanum* species (*S. pinnatisectum*, *S. bulbocastanum)*. Sources of etioled protoplasts were hypocotyl seedlings of *Brassica oleracea* (cabbage, cauliflower) and callus protoplasts of *Cucumis melo*.

Protoplast Isolation

Enzymatic mixture of 1% Cellulase Onozuka R-10 and 0.25% Macerozyme R-10 in rinsing solution Pgly/W5 (Debeaujon and Branchard, 1992, Bříza and Machová, 1991) was used for isolation of mesophyll and hypocotyl protoplasts. Enzymatic mixture of 2% Cellulase Onozuka R-10 and 1% Macerozyme R-10 in rinsing solution Pgly was used for isolation of callus protoplasts (Table 1). Leaves, hypocotyls or calli were segmented and put with enzymatic solution into a thermostat at 25°C for 16-18 hours. Protoplasts were isolated using a conventional method (filtration, centrifugation and purification with 20% sucrose gradient).

Purified protoplasts were rinsed with pre-fusion solution (0.4 M manitol and 1 mM $CaCl_2$) and fusion solution (0.4 M manitol), after centrifugation their required density ($10^5 - 10^6$ protoplasts per 1 ml) was obtained by dilution with fusion solution. The viability of isolated protoplasts used for electrofusions was more than 80% (determined using FDA, Fig. 1).

Protoplast Fusion by Electrific Field

Fusing partner components were mixed in the ratio of 1:1. There were fused protoplasts of *Brassica* genus: mesophyll and hypocotyl, *Cucumis* genus: mesophyll and callus, *Solanum* genus: mesophyll and mesophyll. Protoplast fusion was performed using apparatus ECM 2001 and in electroporation chamber for 20-30 µl with 0.5 mm electrode distance. The following parameters were used for fusions: for protoplast alignment (Fig. 2) - 5V AC and 1 MHz frequency with individual duration of action, for fusion – 1 pulse 10V DC of length 80 µs (Navratilová and Greplová, 2003). Protoplast activity in electric field was observed in invert microscope.

Protoplast Culture

Liquid media (SW11 and LCM1) and solidified medium (SW11) by agarose have been used. Liquid media were maintained in a thin layer on the bottom of a Petri dish (30 mm). Solidified medium contained embedded protoplasts. Protoplasts were cultivated in the dark for 2 weeks at 25°C. After this period, the cultivation continued in a growth chamber with day/night 16/8 hrs cycles at the temperature 22 ± 2°C.

RESULTS AND DISCUSSION

Used concentrations of enzymes, the yield of protoplasts per gram of fresh mass and the viability (determined by FDA) were compared with those used by other researchers (Gajdová et al., 2004). Protoplasts were viable and undamaged following electrofusions. Suspension of protoplasts with the fusion solution was mixed with culturing medium in 1:1 ratio. Homofusion and heterofusion products and also unfused protoplasts could be observed (Fig. 3). The hybrid products of vision (mesophyll + hypocotyl, mesophyll + callus) contained rich vacuole system and many chloroplasts, i.e. characters of both fusion partners.

Protoplasts of *Brassica* genus (Fig. 4) and *Cucumis* genus were subsequently cultured in liquid medium LCM1 (Debeaujon and Branchard, 1992). Protoplasts of *Solanum* genus were mixed with medium SW_{11} (Bříza and Machová, 1991) in double strength all components except mannitol with agarose and later were spread by liquid SW_{11} medium. The culturing in the dark in a thermostat at 25°C continued for 2 weeks. Regeneration of cell walls was recorded and first division was found in 6-8 days of culture (Fig. 5a-c). In *Solanum* genus microcolonies were found in 15 days and microcalli in 22 days (Figs. 6 and 7). The experiments have not been finished yet. Liquid medium was changed in 7-day intervals with lower level of osmoticum.

Techniques for electrofusions of protoplasts from various plant genera (*Brassica, Cucumis* and *Solanum*) and various plant tissues (mesophyll, hypocotyl and callus) were developed. Future research will be focussed on improving of the regeneration from obtained microcalli and calli.

ACKNOWLEDGEMENTS

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Tables

Table 1. Composition of used solutions and media.

Genus	Explant	Enzyme solution	Wash medium	Culture medium
Brassica	mesophyll, hypocotyl	1% cellulase 0.25% macerozyme	W5	SW11
Cucumis	mesophyll	1% cellulase 0.25% macerozyme	Pgly	LCM1
Cucumis	callus	2% cellulase 1% macerozyme	Pgly	LCM1
Solanum	mesophyll	1% cellulase 0.25% macerozyme	W5	SW11

Figures

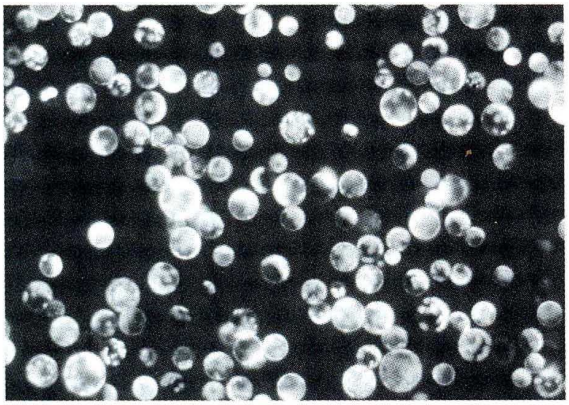


Fig. 1. Protoplasts isolates using FDA.

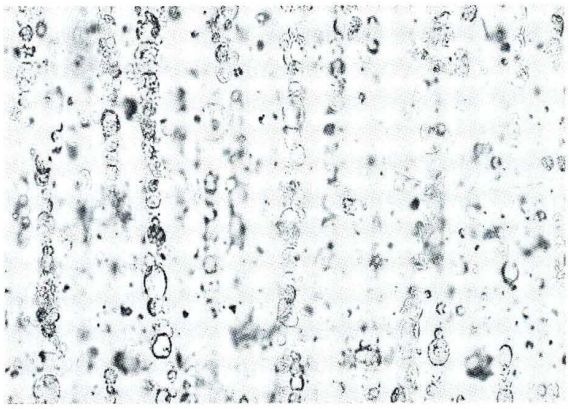


Fig. 2. Protoplast alignment.

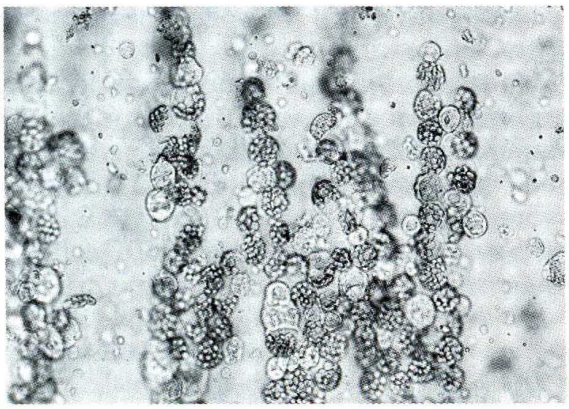


Fig. 3. Homofusion and heterofusion products and unfused protoplasts.

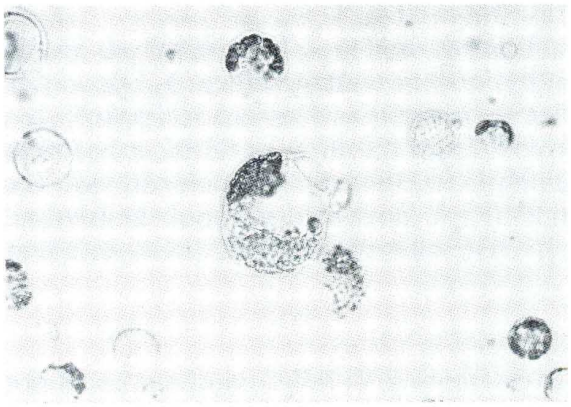
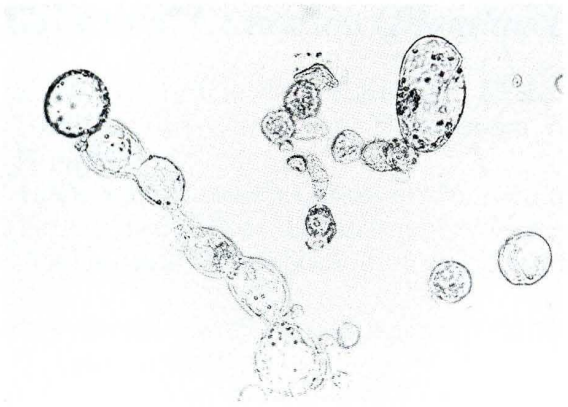
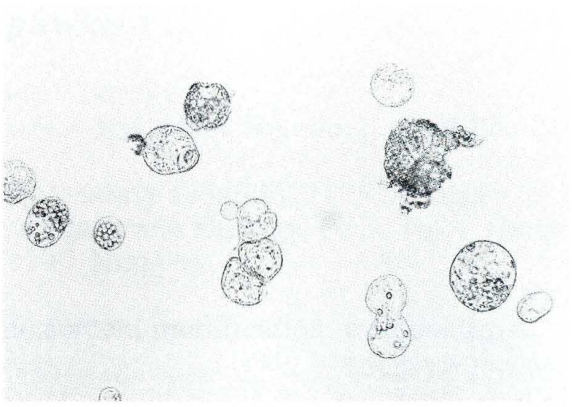


Fig. 4. Protoplasts of *Brassica* genus.



a



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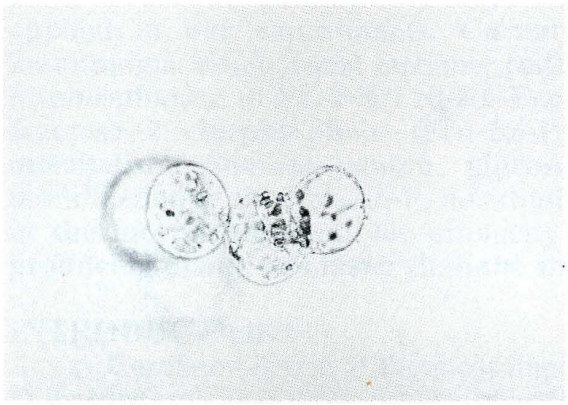

c

Fig. 5. Protoplasts after 6-day-culture.
Cucumis metuliferus 586 (mesophyll) + *C. melo* 1114 (callus).
Brassica oleracea Tolero (mesophyll) + *Brassica oleracea* JQ (hypocotyl).
Cucumis melo 600 (mesophyll) + *C. melo* 1114 (callus).

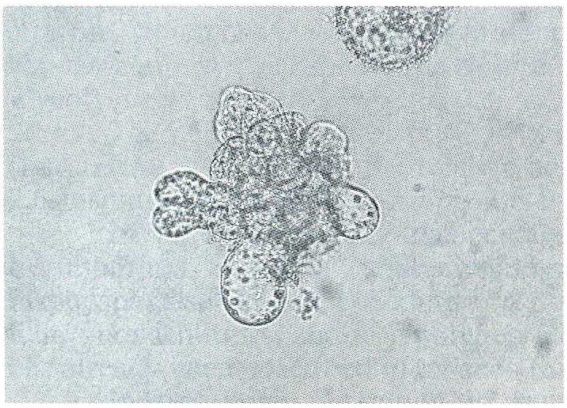

Fig. 6. Microcallus of *Solanum bulbocastanum* 8003 + dihaploid *S. tuberosum* 243.

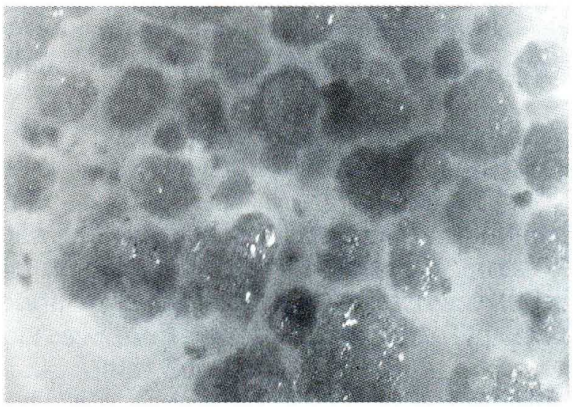

Fig. 7. Calli (dihaploid *S. tuberosum* 299 and *S. pinnatisectum*).

3.4. Contribution to the *in vitro* selection using the protoplast culture technique and proteomic analytical methods

3.4.1. Klíma M., Vítámvás P., Zelenková S., Vyvadilová M., Prášil I.T. (2010): Dehydrin and proline content under cold stress at two irradiation intensities in *Brassica napus* and *B. carinata*. Biol. Plant. (*in press*).

BIOLOGIA PLANTARUM

BRIEF COMMUNICATION

Dehydrin and proline content under cold stress at two irradiation intensities in *Brassica napus* and *B. carinata*

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Abstract

The accumulation of cold-induced dehydrin and proline was related to the level of frost tolerance (FT) in several *Brassica* spp. cultivars. A ~ 47 kDa dehydrin was detected in the leaves of an Ethiopian mustard (*B. carinata*) cultivar and a pair of dehydrins of similar molecular weight in the three (two winter, one spring) oilseed rape (*B. napus*) cultivars, when plants were maintained at 4 °C for one-month under two different light intensities. More dehydrin was accumulated in oilseed rape than in Ethiopian mustard under the high light regime. A significant correlation was observed between leaf dehydrin content and FT and no relationship between proline content and the level of FT or between the levels of leaf proline and dehydrin. Protoplast-derived callus cells behaved differently from leaves sampled from intact plants, as they did not accumulate dehydrin and proline in response to cold stress. The FT of both *B. napus* and *B. carinata* can probably be predicted from the plants' ability to accumulate dehydrin when cold-stressed, and particular cold-induced dehydrins have potential as protein markers improved FT.

Additional key words: Ethiopian mustard, frost tolerance, oilseed rape, protein marker, protoplast-derived calli

Abbreviations: CNT - count unit; FT - frost tolerance; FW - fresh weight; HI - higher irradiation intensity (400 μmol m^{-2} s^{-1}); kDa - molecular mass; LI - lower irradiation intensity (120 μmol m^{-2} s^{-1}); LT_{50} - lethal temperature; PD - protoplast-derived; SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

Assessing the frost tolerance (FT) of *Brassica* crop species under field conditions is hindered by climatic unpredictability, since a mild winter will prevent the differentiation between tolerant and non-tolerant types. Thus, conventional plant breeding has only enjoyed limited success in improving the level of crop FT (Thomashow 1990). A more reliable screening method is clearly needed. A significant research effort has been invested in winter cereals into investigating the efficacy of various cryoprotective compounds, in particular those COR/LEA (COld-Regulated/Late Embryogenesis Abundant) proteins which belong to the dehydrin family. These proteins help to limit water loss from the cell (Kosová *et al*. 2007), probably because they are effective at immobilizing water (Ingram and Bartels 1996). The timing of induction, maintenance and turning off of dehydrin expression, and its resulting accumulation *in planta* during the cold acclimation process is positively correlated with FT (Sarhan *et al*. 1997, Bravo *et al*. 1999, Renault *et al*. 2005). In a comparison based on cold-stressed plants of the contrasting winter wheat cvs. Mironovskaya 808 and Bezostaya 1, Vítámvás *et al*. (2007) noted that FT was predictable on the basis of the accumulation of WCS120 dehydrins. As a result, dehydrin content has been proposed as a marker for FT (Kosová *et al*. 2007). In cell cultures of both blue alfalfa (Parmentier-Line *et al*. 2002) and blueberry (Wolfraim *et al*. 1993), dehydrin content was enhanced by the imposition of a cold treatment. Proline, which has been associated with the general stress response (Kavi Kishor *et al*. 2005, Toka *et al*. 2010, Kumar *et al*. 2010, Li *et al*. 2010), may also be cryo-protective, since proline over-producers in various crops display an enhanced cold tolerance (Patton *et al*. 2007, McClinchey and Kott 2008, Dörffling *et al*. 2009, Pociecha *et al*. 2009, Gothandam *et al*. 2010). The transgenic embryonal masses of hybrid larch (*Larix* × *leptoeuropaea*) with increased levels of proline were significantly more resistant to cold, salt, and freezing stresses (Gleeson *et al*. 2005).

The present research was focused on establishing the relationships between FT and the level of both dehydrin and proline in the leaves of various *Brassica* species, subjected to cold and two different light regimes. We also sought to determine whether these relationships also apply in protoplast-derived (PD) callus cells.

The set of plant material consisted of two winter oilseed rape (*B. napus*) cultivars (Californium and Viking), one spring oilseed rape cultivar (Topas) and doubled haploid line derived from Ethiopian mustard (*B. carinata* A. Braun) cultivar Dodolla. All seedlings were grown for 30 days under a day/night temperature of 22/20 °C and a 12 h photoperiod of intensity 270 μmol m^{-2} s^{-1}, at which point they had reached the four leaf stage. The plants were then divided into two groups. All plants were then exposed for one month to 4 ± 1 °C and a 12 h photoperiod, with one group being given 120 μmol m^{-2} s^{-1} of light (LI), and the other 400 μmol m^{-2} s^{-1} (HI). Both before and after the cold treatment, the last fully developed leaf was harvested from each plant and used to assess proline and dehydrin content.

Protoplast-derived calli were prepared following Kaur *et al.* (2006). When the calli had reached a diameter of 5-8 mm after culturing on medium E, they were divided into two groups of 20 calli. The calli in one group was weighed immediately, and those in the second group were transferred to fresh medium E and weighed after a one-month exposure to 4 ± 1 °C in the dark.

FT was evaluated after the end of the cold treatment. The level of seedling FT was determined by the electrolyte leakage method, as described by Prášil and Zámečník (1998), in which leaf discs are cooled gradually from -3 °C to -30 °C. A software package developed by Janáček and Prášil (1991) was used to calculate the temperature, which was lethal for 50 % of the plants (LT_{50} value).

Proline content was determined according the spectrophotometric Jiménez *et al.* (2006) method. The reaction was monitored at 520nm, using a 1mM proline solution as a standard (*Sigma-Aldrich*, St. Louis, Missouri, USA). Proteins solubilized by boiling were extracted according to Vítámvás and Prášil (2008). Prior to the analysis, pelleted proteins extracted from 60 mg fresh tissue were dissolved in 50 mm^3 of sample buffer, from which 5 mm^3 was taken as a sample for 10 % SDS-PAGE (Laemmli, 1970) separation.

Thermostable proteins were detected by silver staining, and dehydrins by Western blot analysis, according to Vítámvás and Prášil (2008). The molecular weight of the dehydrins was estimated from the gel migration of molecular weight standards (*Bio-Rad Laboratories,* Hercules, California, USA), and their quantity was expressed in the form of a count unit (CNT) per mm^2, by means of the software package Quantity One 4.6.7 (*Bio-Rad*). Individual readings were corrected according to measured differences between duplicate internal standards.

Three independent biological replicates and at least two technical replicates were performed. The *STATISTICA* v7.0 for Windows software package (*StatSoft*, Tulsa, Oklahoma, USA) was used to calculate correlations, simple linear regressions and multi-factorial analyses of variance. Fisher's least significant difference (LSD) was applied to discriminate between treatment means.

Dehydrins of molecular weight ~ 47 kDa were detected in the leaves of all the cold treated plants (Fig. 1). Two similar-sized proteins were present in all three oilseed rape cultivars, while the Ethiopian mustard entry only produced one. Each of the main effects (cold treatment, light intensity, genotype) and the interaction between genotype and light intensity had a significant ($P < 0.05$) effect on dehydrin accumulation. The most effective accumulator of dehydrin was cv. Topas under HI (CNT mm^{-2} 1218.2), followed by cv. Californium HI (919.5), cv. Viking HI (872.1), cv. Californium LI (752.2), cv. Viking LI (707.5), cv. Topas LI (492.3), cv. Dodolla LI (346.8) and cv. Dodolla HI (269.0) (Figure 2*A*).

Both light intensity and genotype had a significant effect on FT, but there was no significant genotype x light intensity interaction. The highest level of FT was achieved by cv. Topas HI (LT_{50} -12.6 °C), followed by cv. Californium HI (-11.4 °C), cv. Viking HI (-10.7 °C), cv. Californium LI (-10.2 °C), cv. Viking LI (-10.0 °C), cv. Topas LI (-9.8 °C), cv. Dodolla LI (-4.9 °C) and cv. Dodolla HI (-4.5 °C) (Figure 2*B*).

The correlation between the content of dehydrin in the leaves and the LT_{50} was highly significant ($P \leq 0.05$) and negative (-0.87, Fig. 2*C*). Within the HI treatment, the correlation was even higher (-0.95), while within the LI treatment, it was lower but still significant (-0.75, Fig. 2*C*). Because a higher FT is reflected by a more negative value of LT_{50}, this negative correlation implies a positive one between dehydrin content and FT. The experiments showed that the dehydrin content of the leaves, whether the plants were exposed to LI or HI, could be used to rank the entries with respect to FT.

Cold treated plants of three of the four entries (excluding cv. Topas) accumulated the same similar or less proline than the non-treated ones. Thus non-treated cv.Viking accumulated 142.3 nmol proline per g fresh weight, but only 88.7 and 93.3 nmol in, respectively, the cold-treated plants exposed to LI and HI. Thus there was no relationship between proline content and the level of FT (Figure 2*D*) or between the levels of leaf proline and dehydrin.

The effects on dehydrin content of cold treatment, genotype and the cold treatment × genotype interaction were all non-significant for the protoplast-derived calli. Dehydrin proteins were present in the SDS-PAGE profiles of some of the calli not exposed to cold treatment, but not in any of their cold-treated equivalents (Fig. 1). The highest dehydrin content was present in the cold treated calli of cv. Californium (CNT mm^{-2} of 493.3) and in the non-cold treated calli of cv. Viking (437.8). The lowest levels of dehydrin were present in cv. Dodolla calli (non treated: 326.7, cold-treated: 365.5). Similarly, for proline content, there was no significant cold treatment main effect, nor any significant cold treatment × genotype interaction. Although the genotype main effect was significant ($P < 0.05$), the level of proline present in the calli of the non-cold treated plants was mostly higher than in the cold treated ones. Thus the non-treated cv. Dodolla calli accumulated 181.0 nmol proline per g fresh weight, but the

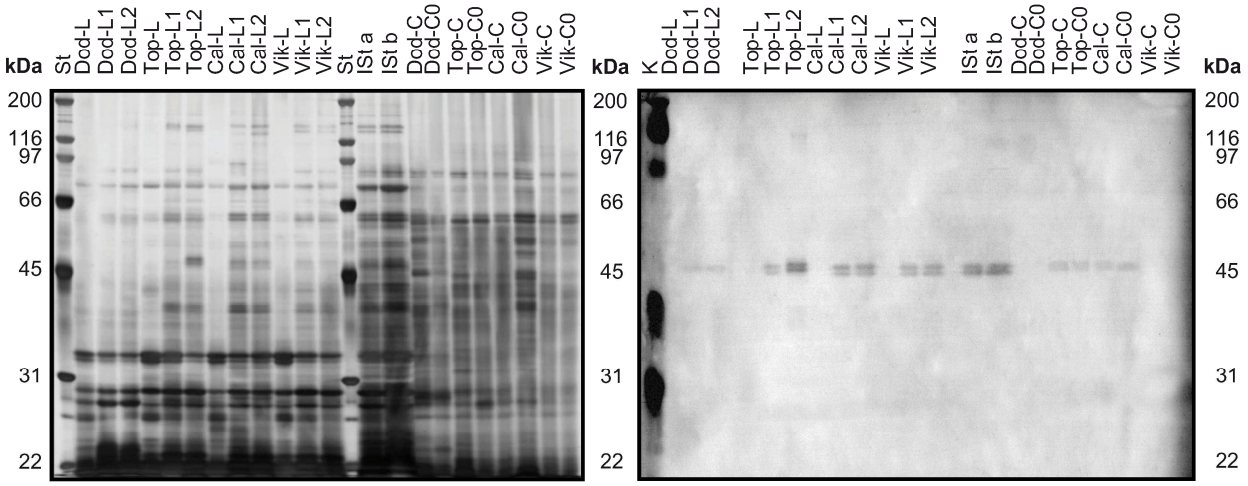

Figure 1. SDS-PAGE profile (left) and Western blot probed with an anti-dehydrin antibody (right) derived from extracts of leaf and protoplast-derived callus. St, molecular weight standards; K, kaleidoscope broad range pre-stained standards (Bio-Rad); ISt a and ISt b, internal standards (b is a 1:1 dilution of a; a is an extract from cv. Californium plants held for 60 days at 4 °C under HI); Dod, cv. Dodolla; Top, cv. Topas; Cal, cv. Californium, Vik, cv. Viking; L, leaf extract; C, callus extract; 0, cold treatment in the dark; 1, cold treatment under LI; 2, cold treatment under HI; unnumbered, no cold treatment.

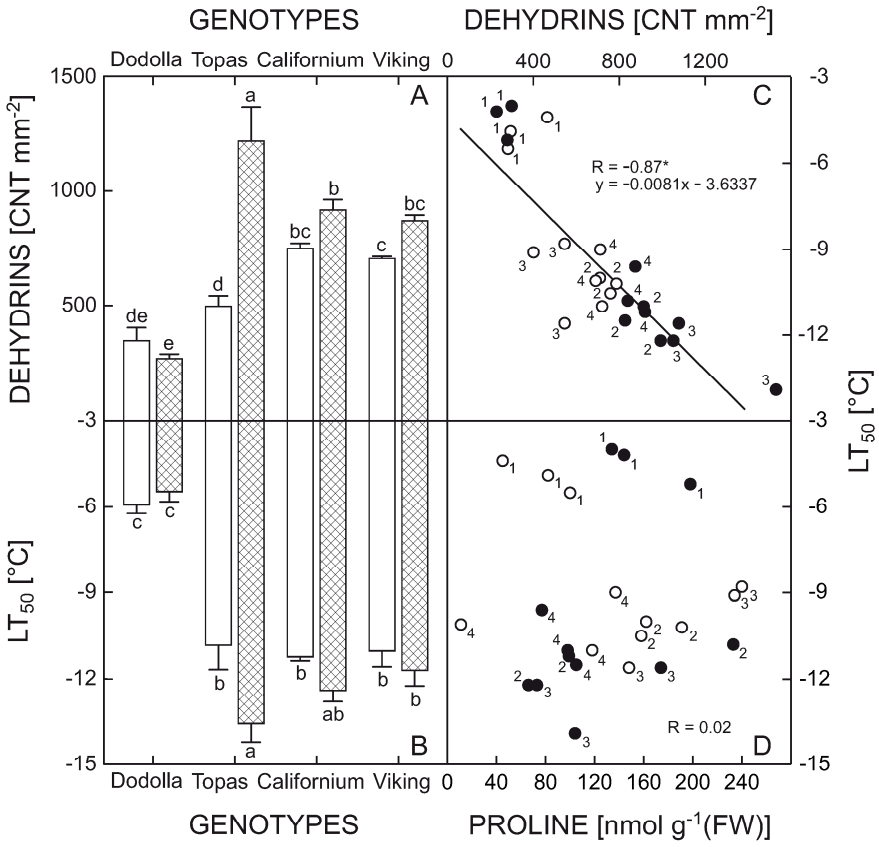

Figure. 2. Dehydrin content (*A*), the level of FT (*B*), and the relationship between FT and dehydrin (*C*) or proline (*D*) leaf content of plants exposed to cold for one month under two different light intensities. In *A* and *B*, the columns denote means ± standard error; means labelled with the same lower case letter do not differ significantly from one another ($P = 0.05$). In *C* and *D*, circles denote values for each biological replicate. * - $P < 0.05$. Empty columns and circles refer to the LI treatment, hatched columns and filled circles to the HI treatment. 1, cv. Dodolla; 2, cv. Californium; 3, cv. Topas; 4, cv. Viking.

cold treated ones accumulated only 29.3 nmol. No correlation between proline and dehydrin content in the calli was evident, and the behaviour of the calli was overall too dissimilar to that of the leaves to contemplate an *in vitro* test of FT.

Other studies of dehydrin induction in cold treated oilseed rape have identified a 31 kDa ERD10 dehydrin (Deng *et al.* 2005) and a 19.2 kDa BnDHN1 (Yao *et al.* 2005), but the ~ 47 kDa products detected in the present experiments have not been previously observed. Light intensity during frost hardening was thought to be an important factor in the development of FT in winter rye and wheat, since hardening under low light conditions was rather less effective than under normal light conditions (Gray *et al.* 1997, Janda *et al.* 2007).

A similar situation obtained here with oilseed rape, while the somewhat reduced FT (and leaf dehydrin content) of the Ethiopian mustard cultivar in response to higher light intensity was likely an effect of photoinhibition. A light intensity of 400μmol m^{-2} s^{-1} combined with a low temperature may well have compromised the activity of the photosynthetic centres, leading to some inhibition of overall metabolism, and hence of FT. On the other hand, the greater FT and higher leaf dehydrin content of the spring oilseed rape cultivar Topas could be explained by the fact that this cultivar appeared to be more capable than were the winter ones of exploiting higher light intensities.

The lesser FT of the latter may also reflect the longer period needed by winter oilseed rape to acquire FT, a difference noted in the cereals by Fowler (2008). Different rates of FT development, as well as differences in the levels of dehydrin expression and accumulation, may be a pleiotropic effect of vernalization genes, as suggested by Kosová *et al.* (2007). Although Rapacz (1999) also showed that the FT of spring oilseed rape cultivars was comparable to that of winter ones, this does not necessarily imply that spring types become as (or even more) winter hardened than winter ones.

When a plant reaches its reproductive stage, its ability to achieve a high level of FT becomes substantially reduced. In vernalization requiring plants, this stage is reached only once the plant's vernalization (as well as any photoperiod) requirement has been fulfilled, whereas in spring types, the timing of the switch depends on the rate of the plant's development. Thus, unlike spring types, winter ones are able to re-acclimate during the winter season before they have reached a relatively advanced stage of development (Prášil *et al.* 2005). The ability to re-acclimate is usually an important factor for successful over-wintering.

The relationship between leaf dehydrin content and the level of FT we have reported agrees well with what has been observed for barley (Kosová *et al.* 2008, 2010), wheat (Vítámvás *et al.* 2007, 2010) and olive (Cansev *et al.* 2009). Although proline was not significantly accumulated as a result of cold treatment in our experiments, the literature contains a number of reports that cold treatment does induce the accumulation of proline (Atici *et al.* 2003) and that there is a positive correlation between the level of proline in the leaf and FT (McClinchey and Kott 2008, Dörffling *et al.* 2009, Walker *et al.* 2010). However, in cauliflower, Fuller *et al.* (2006) suggested the opposite trend, specifically that FT was associated with lower proline concentrations. Janská *et al.* (2010) have described an ambiguous relationship between the proline content of *in vitro* selected hydroxyproline resistant winter oilseed rape and FT, with some lines accumulating less proline after cold treatment than when not exposed to any cold treatment. Bhattarai and Fettig (2005) have suggested that dehydrin accumulation forms part of the response of many plant species to various stresses. Thus the presence of dehydrins (and the elevated levels of proline) in the non-cold treated calli is indicative that other stress factors were probably operating in these cell lines. In blueberry cell cultures, Parmentier-Line *et al.* (2002) have shown that a number of dehydrins are produced under supposedly non-stressful conditions. Our findings agree with those of Parmentier-Line *et al.* (2002), who showed that the responses of *in vitro* grown cells was very different from that of whole plants, thus making cell culture unsuitable for the study of dehydrin expression in whole plants.

We conclude that FT in oilseed rape and Ethiopian mustard can be predicted on the basis of the dehydrin content of the leaves of cold treated plants, and that there is potential to develop cold-induced dehydrins as protein markers for the improvement of FT in *Brassica* spp.

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4. Discussion and Conclusions

The Thesis deals with the protoplast culture technique and its application in protoplast fusion and *in vitro* selection in some genotypes of the family Brassicaceae. The main aims were to establish and optimise the isolation, chemical fusion and electrofusion of protoplasts and further regeneration in the protoplast culture. The next step was aimed at the use of protoplast culture technique for *in vitro* selection and identification of desired genotypes by means of proteomic and analytical methods.

Several members of the genus *Brassica* belong to the most important economic crops worldwide. Therefore, great attention has been paid to continuous improvement of these species. As a result of a long-term breeding for agronomically important traits, the disappearance of other important characters has taken place in many crops. These include resistance to important and/or newly emerging diseases (or pests as well), the specific composition of fatty acids in the oil, or other characters that can be potentially useful in the industry, pharmacy, etc.

Even if some of these traits can be transferred from their close or distant relatives by using traditional methods of intra-, interspecific or distant (wide) hybridization, these methods are often laborious and time consuming. In addition, in some traits it is difficult or even impossible considerable improvement by means of classical methods of breeding.

For this reason, plant breeders, in collaboration with scientists have tried to use biotechnological methods which could overcome these barriers and broaden the genetic diversity. One of these procedures is somatic hybridization, facilitated by fusion of isolated protoplasts and subsequent regeneration of the desired genotypes in protoplast cultures. Although there have been obtained intraspecific, interspecific and intertribal somatic hybrids within the family Brassicaceae, their wider use in breeding usually impede both low vitality and fertility. It is therefore necessary to optimise methods of protoplast culture and somatic hybridization in particular genotypes, which can be used as initial materials for the substantial improvement of cruciferous crops.

In our experiments, efficient and reliable protocols for protoplast isolation, electrofusion, chemical fusion and protoplast cultivation of mesophyll and hypocotyl protoplasts of selected *Brassica* genotypes have been developed. Regeneration of the cell wall was achieved in the majority of examined genotypes; strong effect of the genotype on dividing of cells, microcallus formation and callus regeneration was confirmed by means of statistical analysis. There were remarkable differences between particular genotypes not only in the productivity (i.e. in the number of microcalli per cultivated protoplasts) but also in the morphological and physiological characteristics of obtained microcalli. However, significant differences were detected also between biological replicates within some genotypes; this phenomenon might be caused by certain level of the hetero-

geneity of biological material used for successive replications. Finally, more than 300 *B. oleracea*, *B. napus* and *B. carinata* regenerants were transferred into soil, and they produced normal inflorescences and set seeds.

In the experiments with protoplasts of *B. oleracea* and *B. napus*, minimum duration of enzyme treatment time and prolonged dark period in the early stages of protoplast culture increased the viability of protoplasts and thus the regeneration process. In the previous findings in *Brassica* vegetables either the viability was low or the regeneration percentage decreased (Glimelius 1984, Kirti *et al.* 2001). The isolation time of protoplasts in an enzyme solution could affect not only the viability of protoplasts but also the nature of plasmalemma (Pilet 1985), affecting the wall biosynthesis and thus the division process.

Culture density has a fundamental effect on the plating efficiency of protoplasts. In concordance with the results of Chuong *et al.* (1985), in our experiments was found that with higher culture densities higher plating efficiency could be achieved. For mesophyll protoplasts, Vamling and Glimelius (1990) also recommended a higher concentration of protoplasts. In the initial culture medium an equal amount of 6-Benzylamino purine (BAP) and 1-Naphthaleneacetic acid (NAA) in the presence of a low amount of 2,4-Dichlorophenoxyacetic acid (2,4-D) was favourable in our experiments. Dietert *et al.* (1982) also pointed to the better growth of callus in the presence of a low concentration of 2,4-D. However, according to Glimelius (1984) and Kohlenbach *et al.* (1982) a high amount of 2,4-D is essential for cell division and callus proliferation. No such effect was evident in our experiments. It might be linked to the endogenous level of auxins in the used plant material.

A prolonged dark period is essential for the stability of protoplasts and thus for the formation of microcalli. In the presence of light H^+ ion extrusion takes place, which increases the acidity of culture medium (Schubert and Matzke 1985). Cleland (1975) showed that H^+ ion extrusion was enhanced by the action of auxins. Under illumination the cultures turn brown, which affects the division efficiency of protoplasts. In our experiments not even after one month of culture was there a sign of browning in the cultures which were transferred to light after microcalli had been formed.

Differentiation or induction medium is a critical part of the protoplast culture protocol. Based on the results it can be concluded that cytokinin 2-isopentenyl adenine (2iP) is more efficient than BAP in the induction of regeneration of calli. After one month on the induction medium, further transfer to regeneration medium F and MS without growth regulators showed no profound effect on regeneration frequency. The differentiation of organs was found to take place on medium E. Development of fragile calluses is in accordance with the reports by Kirti and Chopra (1990) in *B. juncea* and Kohlenbach *et al.* (1982) in *B. napus*.

The absence of root development in almost 50% of regenerated shoots in *B. napus* is comparable to the results of Hu *et al.* (1999) and is genotype dependent. In our experiments, *B. oleracea* showed a high division frequency in medium B in comparison with medium KM8p (Kao and Michayluk 1975), which corresponded to protoplast division in some haploid lines generated from a microspore culture of *B. carinata*. There are some variations in growth regulator concentration requirements for different subspecies (Robertson *et al.* 1988, Jourdan *et al.* 1990, Kirti *et al.* 2001). In the present liquid culture system it is easy to handle the calli, and the medium described by Pelletier *et al.* (1983) proved to be quite suitable for *B. oleracea* protoplast culture.

Although the formation of cell divisions and microcallus structures were detected almost in all genotypes of *B. napus* and *B. carinata* and the progress of the development was similar to the results, published before (Glimelius 1984, Hu *et al.* 1999, Chen *et al.* 2004), there were remarkable differences between particular genotypes not only in the productivity (i.e. in the number of microcalli per cultivated protoplasts) but also in the morphological and physiological characteristics of obtained microcalli. While the genotype BC-6 proved to be the most productive (31.4×10^{-2}% of regenerated microcalli per cultivated protoplasts), genotypes BN-OP-01 and BN-SL-03/04 demonstrated rather low level of regeneration ability. In addition, above mentioned, low productive genotypes often formed microcalli that gradually turned brown at early stages of development. Such structures may exude various substances, which can negatively affect the development of other, normally dividing microcalli (Chen *et al.* 2004). Strong impact of the genotype on the initialization of cell divisions and further development of dedifferentiated tissue in the protoplast culture was presented previously by various authors, namely Hu *et al.* (1999) in *B. napus* and *B. juncea*; Chen *et al.* (2004) in *B. oleracea*; Narasimhulu *et al.* (1992) in *B. carinata.*

Successful conversion of microcalli to calli was observed almost in all tested genotypes; however, outstanding differences in regeneration ability were detected not only between individual genotypes but also between some biological replicates within particular genotypes. The relevance of both factors was later confirmed via statistical analyses of measured data. Moreover, in some biological and technical replicates no regeneration of calli, characterised by formation of prolonged cells, was detected, although such genotypes produced satisfactory amount of calluses in other replications. For example, viable calli were regenerated only from one biological replication of the genotype BN-SL-03/04. The differences in the efficiency of the callus formation between genotypes have been reported before, for instance Hu *et al.* (1999).

Significant differences between some biological (i.e. successive) replications of the same genotype can be explained by the heterogeneity of the biological material used for establishing of protoplast cultures and was observed also in the experiments of Chen *et al.* (2004). Plants with properly developed leaves and stems were derived only from

bright green calluses with dark green meristematic structures inside callus tissue. No shoots were regenerated from white and yellow calluses without meristematic zones. In total, only sixteen calluses of genotypes BC-1, BC-6 and BN-OP-01 regenerated shoots; several calli produced more than one shoot. All regenerated plants were characterized by certain level of hyperhydricity; such type of plants was described also by Jourdan and Earle (1989) in the protoplast culture of four *Brassica* species, Hu *et al.* (1999) in *B. napus* and *B. juncea*. This undesirable physiological state was eliminated by repeated subcultivation on MS medium without growth regulators.

Techniques for electrofusion of protoplasts from various genotypes and various plant tissues (mesophyll and hypocotyl) were developed. The highest frequency of fused cells was achieved in a small fusion chamber with 5 V alternate current (AC) for 8–15 seconds to chain protoplasts, followed by one 20 V direct current (DC) pulse of 80µs length for the perforation of the cytoplasmic membrane. Fusion of membranes took place during progressively reduced AC voltage (5 V to 0 V in 5 seconds). Used concentrations of enzymes, the yield of protoplasts per gram of fresh mass and the viability (determined by fluorescein diacetate) were comparable with those published before (Gajdová *et al.* 2004). After fusion, protoplasts in the fusion solution were mixed with the cultivation medium in 1:1 ratio and microscopically analysed. Homofusion and heterofusion products and also unfused protoplasts were observed. The hybrid products of fusion (mesophyll + hypocotyl) contained rich vacuole system and many chloroplasts, i. e. characters of both fusion partners. Protoplast suspensions in the liquid cultivation medium B were maintained in the dark in a thermostat at 25°C. Regeneration of cell walls and first divisions were recorded within 6–8 days of culture. Liquid medium was replaced in 7-day intervals with the one of lower level of osmoticum.

Concerning chemical fusion between *B. napus* and *B. carinata*, it is evident, that only the proper combination of polyethylene glycol (PEG) 6000 (molecular weight) concentrations and time durations can provide satisfactory fusion frequency. From results can be concluded that all the 30% PEG solutions provided good results (about 8% of fusants) together with 25% PEG solution with the period of 20 minutes. The efficiency of these combinations is comparable with the results, obtained by Gürel *et al.* (2002). Both 20% PEG concentrations and shorter period of 25% concentration showed rather low efficiency. However, both combinations with 30% PEG solution increased the number of multifusants, unfavourable for practical applications. Analogously to the production of calli, significant differences between biological replicates in fusion frequency were detected as well. These circumstances might be explained by the heterogeneity of fused material during experiments even if all measurable environmental conditions were maintained. Protoplasts of fused cultures regenerated cell walls and first divisions were observed after the 4^{th} day of the culture. Small calli, approx. 1–2 mm in diameter turned brown-yellow and did not develop desired callus structures.

The development of protoplast technology and fusion between *B. rapa* and *B. carinata* has enabled the production of somatic hybrids between these cruciferous species and the formation of novel cybrids and hybrids (Jaiswal *et al.* 1990). Previous findings of other researchers confirmed that the successful and efficient utilization of protoplast culture technique depends not only on species but even on variety (Power *et al.* 1984, Moreno-Ferrero and Nuez-Vials 1985, Jain *et al.* 1988, Li *et al.* 1999). In our experiments, 58 calli were obtained via protoplasts fusion between *B. rapa* 31/96 and *B. carinata* line 1 with the fusion frequency about 25%. Consequently, 14 calli produced shoots on regeneration media. The differentiation process and shoot regeneration occurred within 3–4 months; similar results were published before (Fahleson *et al.* 1994, Gerdemann-Knörck *et al.* 1995). Fusion and regeneration frequencies were similar to those obtained in hybridization experiments between more closely related species (Fahleson *et al.* 1993).

The three parameters (protoplast viability, yield and frequency of cell division) were used to determine optimal molecular weight of PEG and time duration. Using 30% PEG 6000 for ten minutes was the most effective, while combinations with 30% PEG 6000 for 15 min, 30% PEG 4000 for 15 min and 30% PEG 4000 for 10 minutes reduced viability of protoplasts and cell wall regeneration, cell division and production of microcolonies. These results conform to previously published experiments (Kirti *et al.* 1991, Kirti *et al.* 1992a,b, Narasimhulu *et al.* 1992).

One group of obtained plantlets was morphologically identical with *B. carinata* plants (from the aspect of size and form of leaves) while the rest of regenerants was conformable neither *B. carinata* nor *B. rapa*. The hybridity of plants was confirmed via flow cytometry. Approximately 40% analyzed samples were only *B. carinata* plants, established via fusion within this species and 60% of plantlets obtained via protoplast fusion between *B. carinata* and *B. rapa*. According to our results, both *B. rapa* 31/96 and *B. carinata* line 1 are suitable genotypes for further experiments in protoplast fusion. We identified several genotypes with good regeneration ability and optimal combination between PEG concentration and exposure time to provide satisfactory fusion rate and further regeneration. These findings may help in further experiments, not only in closely related species of the genus *Brassica*.

The assessment of *Brassica* crops for frost tolerance under field conditions is often complicated by mild winters, not allowing effective differentiation of resistant and susceptible cultivars and breeding materials. Thus, traditional plant breeding systems have met with limited success in improving the frost tolerance (FT) of agronomic plants (Thomashow 1990). It is therefore necessary to introduce more accurate methods to ensure the rapid selection of resistant genotypes, enabling early application by the use of *in vitro* tissue culture systems.

In our experiments, dehydrins of molecular weight ~ 47kDa were detected in protoplast-derived (PD) calli of two *B. napus* genotypes, Topas and Californium. In *B. napus*, Deng *et al.* (2005) reported one band of dehydrin ERD10 of 31kDa after cold acclimation and Yao *et al.* (2005) BnDHN1 of 19.2kDa. Although we did not observe significantly higher accumulation of proline after cold treatment in general, there are studies revealing a higher content of proline in cold-acclimated leaves of cabbage (Atici *et al.* 2003) or positive correlations between proline levels and improved FT (McClinchey and Kott 2008, Dörffling *et al.* 2009, Walker *et al.* 2010). On the contrary, Fuller *et al.* (2006) detected good resistance to salt and/or frost, but relatively low proline concentrations in several lines of cauliflower. Additionally, one line had a high proline concentration, but no measurable improvement in stress resistance. Moreover, Janská *et al.* (2010) found ambiguous relationships between the proline content of *in vitro* selected hydroxyproline resistant winter oilseed rape and FT and some genotypes had a higher content of proline under control conditions, when compared to plants after 60-day cold acclimation at 4°C.

The presence of dehydrins and high levels of proline even in non-acclimated protoplast-derived calli could be explained by an interaction with additional stress factors, causing dehydration of plant tissues, most probably occurring under *in vitro* conditions of protoplast and callus cultures. Parmentier-Line *et al.* (2002) also published similar results in cell cultures of blueberry, where 65 and 30kDa dehydrins were detected also in non-treated variants. On the other hand, the level of the 30kDa dehydrin increased significantly after only 1 d at 4°C and then increased gradually during the whole period of cold treatment. Bhattarai and Fettig (2005) also claimed that, in many plants, dehydrins are produced during various stress situations. Another possible reason of this phenomenon could be the somaclonal variation, often reported in connection with the regeneration of dedifferentiated tissues *in vitro*. Our findings are in accordance with Parmentier-Line *et al.* (2002), where the results differed in too many ways from those on whole plants and made cell culture unsuitable for the study of dehydrin expression. Our results indicate that the prediction of frost tolerance in *B. napus* and *B. carinata* based on dehydrin or proline detection and quantification in the protoplast-derived calli is not feasible as there was no relationship with levels of these compounds, detected in the leaves.

The results suggest that the routine usage of protoplast culture and somatic hybridization techniques in the breeding procedures of *Brassica* crops could be possible, though requires deeper screening for suitable genotypes with regard to their regeneration ability in the protoplast culture and further optimization of the entire process, involving whole plant regeneration.

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5. Summary (in Czech)

Protoplastové kultury vybraných zástupců čeledi Brassicaceae

Fúze protoplastů a následně protoplastové kultury jsou využívány především pro manipulaci s cizorodými geny s cílem rozšířit genetickou diverzitu. Protoplasty tak mohou být využity ve šlechtitelských programech, zejména pro překonání bariér nekřížitelnosti při tvorbě tzv. somatických hybridů. V této práci byl optimalizován a zaveden efektivní postup izolace, elektrofúzí, chemických fúzí a kultivace protoplastů u vybraných genotypů rodu *Brassica*. Byly stanoveny optimální kombinace doby působení a koncentrace polyetylén glykolu (PEG) a stanovena frekvence fúzí mezi genotypy. 25% PEG, aplikovaný po dobu 20 minut přinesl nejlepší výsledky s ohledem na životnost protoplastů a frekvenci fúzí. Obnova buněčné stěny a tvorba mikrokalusů byla zaznamenána téměř u všech testovaných genotypů. Byl zjištěn signifikantní vliv genotypu na další dělení buněk a vývoj nediferencovaného pletiva. Mikrokalusy byly získány z chemických fúzí mezi *Brassica rapa* a *B. carinata* a mezi *B. carinata* a *B. napus* a celistvé rostliny, získané z protoplastových kultur genotypů *B. oleracea* byly převedeny do nesterilních podmínek a přemnoženy. Byly získány somatické hybridy s dobře vyvinutými pravými listy z chemických fúzí mezi *B. carinata* a *B. rapa*. V dalších fázích experimentů byla po chladovém ovlivnění sledována akumulace dehydrinů a prolinu v kalusech z protoplastových kultur a porovnána se stupněm chladové odolnosti listů zvolených genotypů *Brassica* ssp. I když byly identifikovány nové kandidátní dehydriny u dvou kultivarů, předpoklad predikce chladové odolnosti genotypů na základě stanovení koncentrace dehydrinů a/nebo prolinu v kalusech z protoplastových kultur nebyl potvrzen, protože koncentrace těchto specifických látek v kalusovém pletivu nekorelovala s obsahy, stanovenými v listových pletivech chladově ovlivněných rostlin.

6. Author's Publications

Klíma M., Vítámvás P., Zelenková S., Vyvadilová M., Prášil I.T. (2010): Dehydrin and proline content under cold stress at two irradiation intensities in *Brassica napus* and *B. carinata.* Biol. Plant. (*in press*).

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7. Appendix

Appendix list:

38 Viability check after electrofusion by means of fluorescein diacetate staining

39 Regeneration of the cell wall after electrofusion in the protoplast culture of cabbage cv. Rufus (IAD) and winter rapeseed cv. Cando (UV)

40 The first division after electrofusion in the protoplast culture of cabbage cv. Vysocké (IAD) and winter oilseed rape cv. Caracas (UV)

41 Regeneration after electrofusion: IAD red cabbage cv. Azur × UV winter rapeseed cv. Cando

42 Microcallus after electrofusion of winter rapeseed DH OP3 and Abyssinian mustard (*B. carinata*) BC11

43 Calli after electrofusion – IAD red cabbage cv. Vysocké × UV winter rapeseed cv. Caracas

44 A heterofusant after chemical fusion – cauliflower cv. Fortuna 13 × × cabbage cv. Torpedo

45 Heterofusants after chemical fusion – cabbage DH Vysocké B × × cabbage cv. Torpedo

46 A polyfusant after chemical fusion – cabbage DH Vysocké B × × cabbage cv. Torpedo

47 Dividing of protoplasts after chemical fusion – IAD cabbage cv. Torpedo × UV winter rapeseed cv. OPV-21/55

48 Microcallus regeneration after chemical fusion – IAD cabbage cv. Vysocké × UV winter rapeseed cv. OPV-21/55

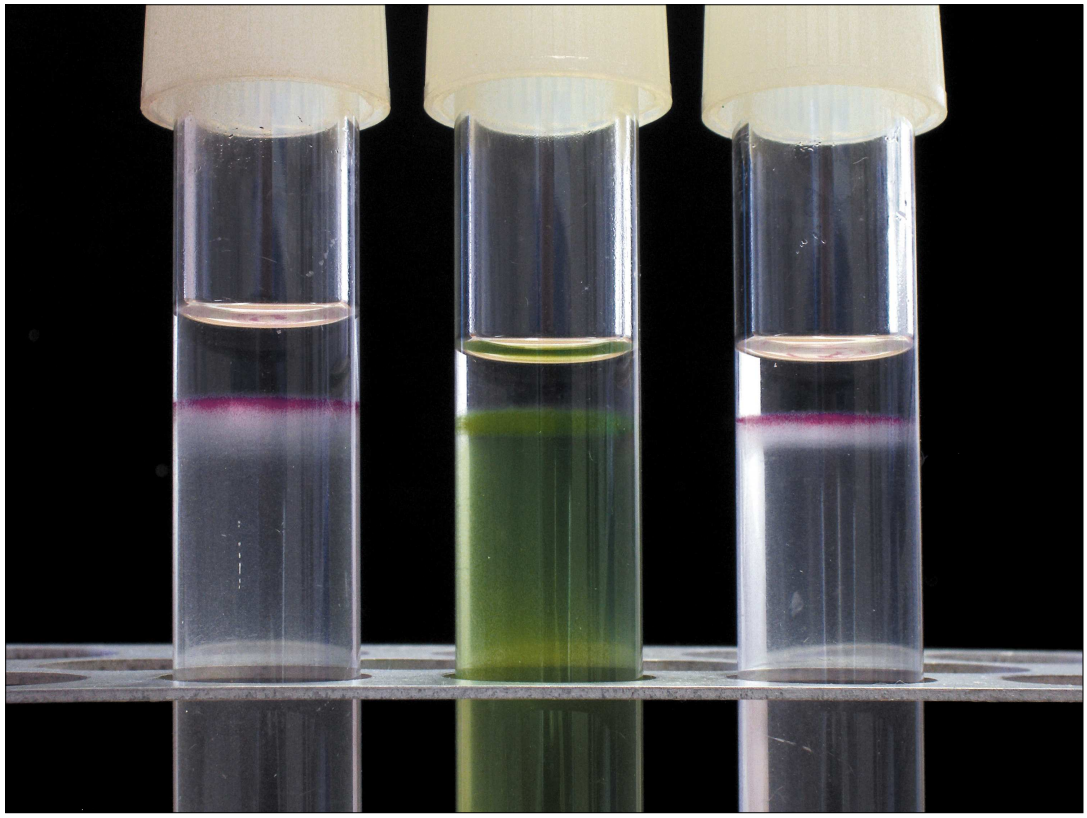
49 Microcalli after chemical fusion – cabbage DH Vysocké B × cabbage cv. Torpedo (45 days of the culture)

50 Microcalli after chemical fusion – cabbage DH Vysocké B × cabbage cv. Torpedo (65 days of the culture)

51 Calli after chemical fusion (cabbage cv. Torpedo × cauliflower cv. Siria)

52 Regeneration after chemical fusion – cabbage DH Vysocké B × × cabbage cv. Torpedo

Figure 1



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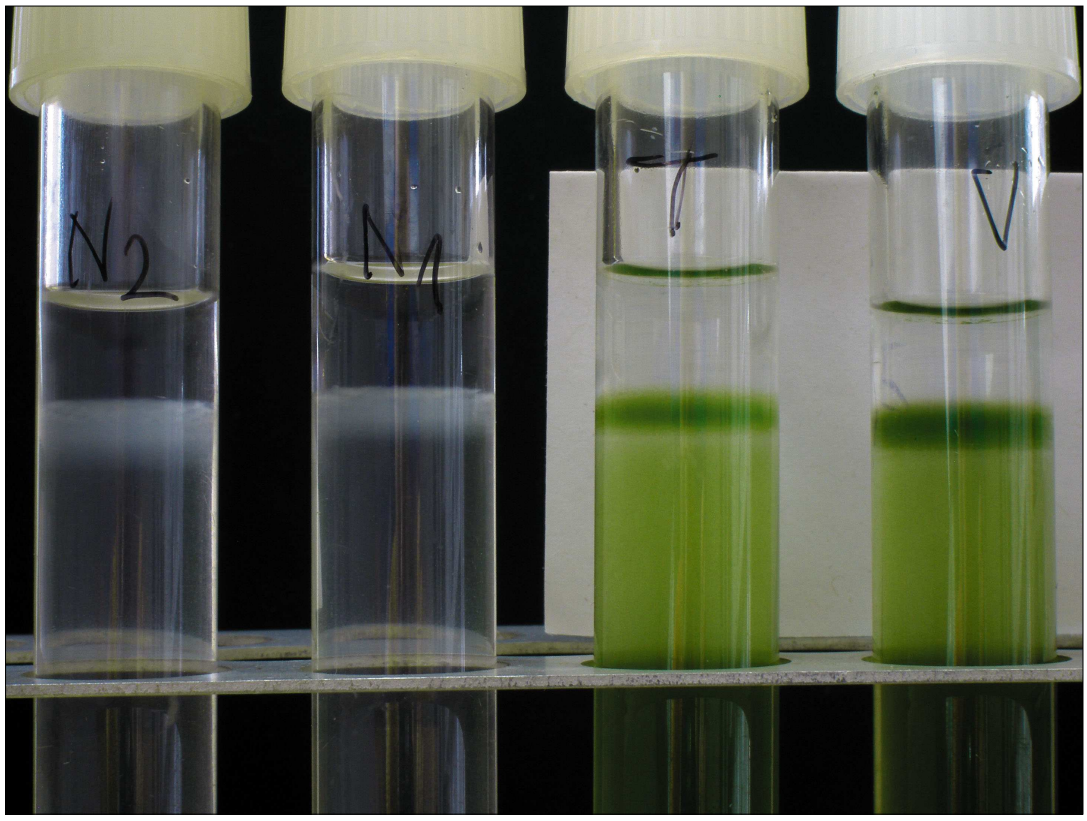
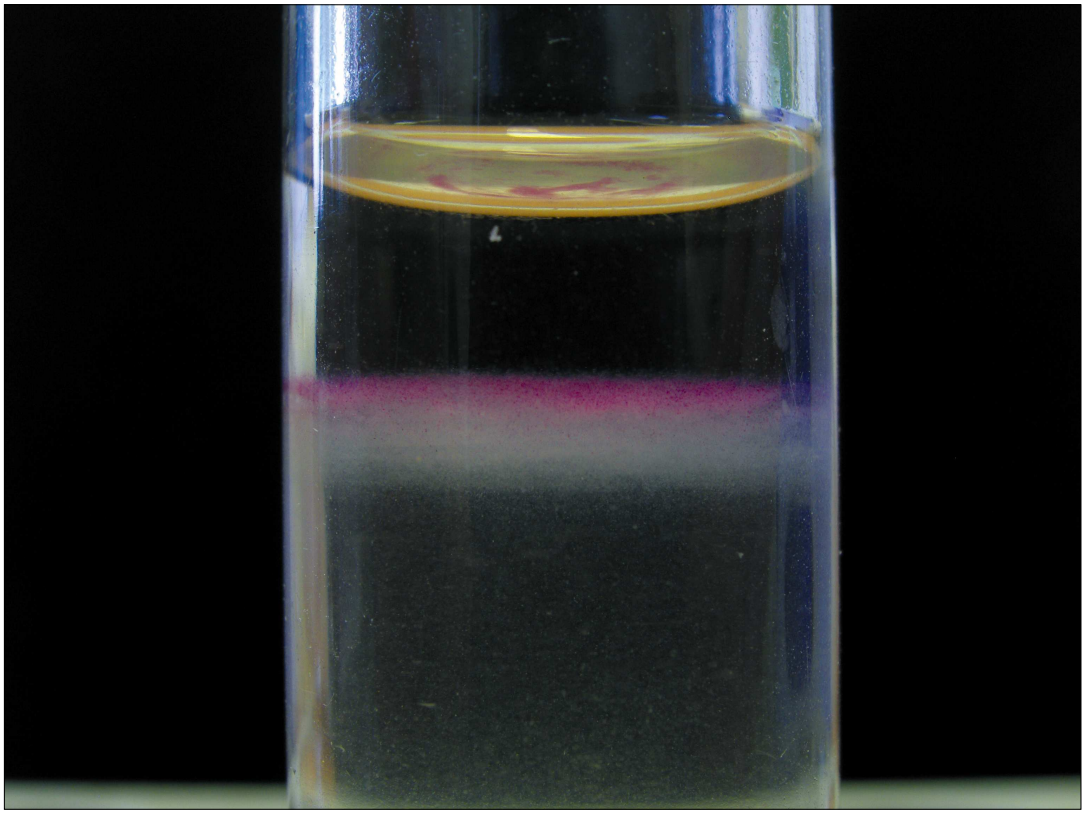


Figure 3



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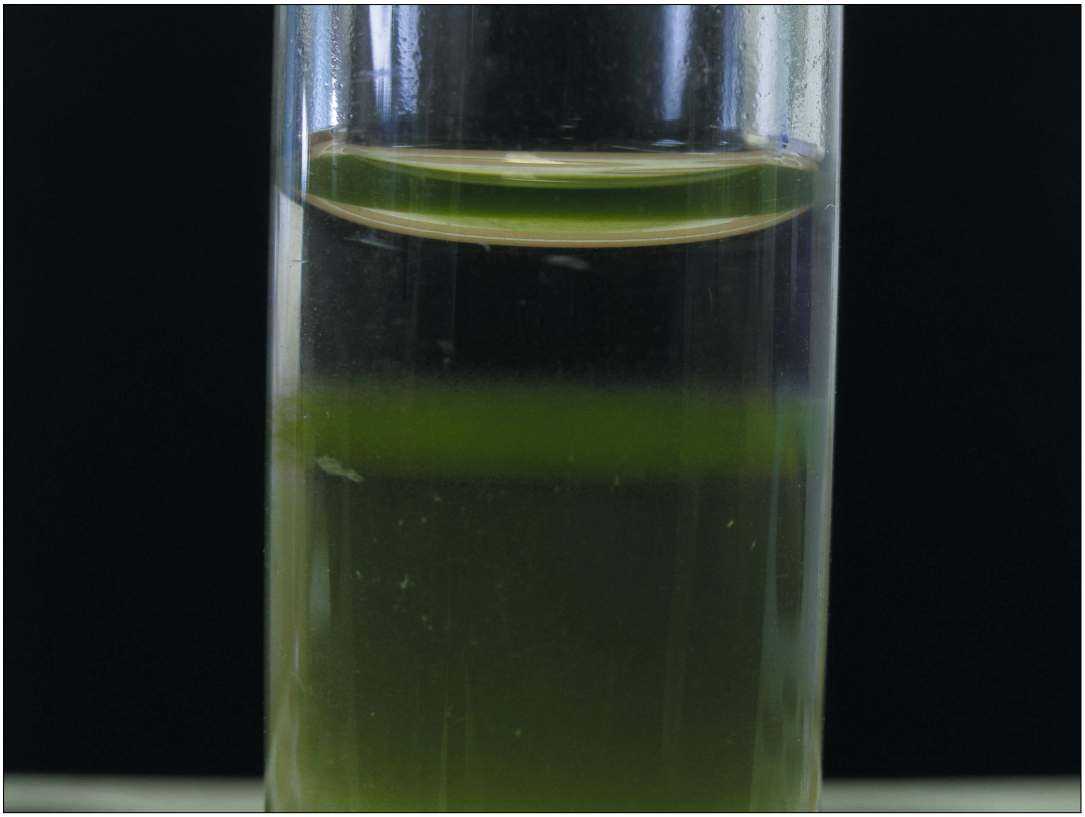
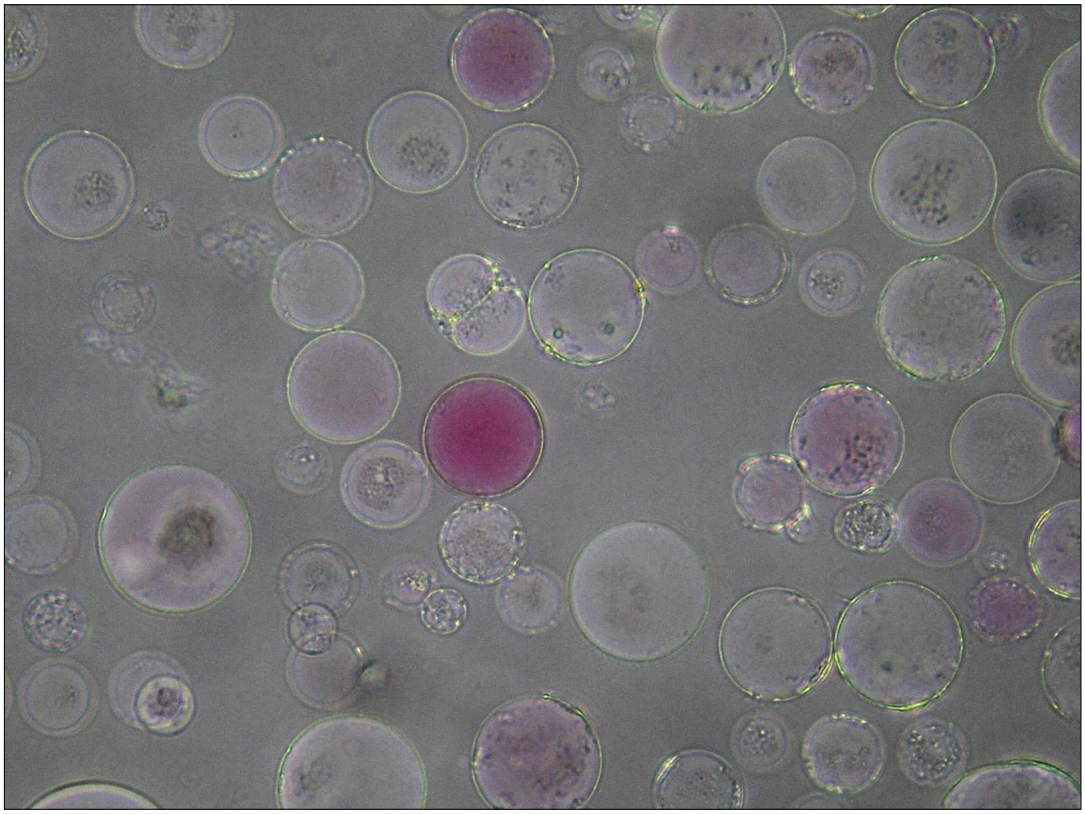


Figure 5



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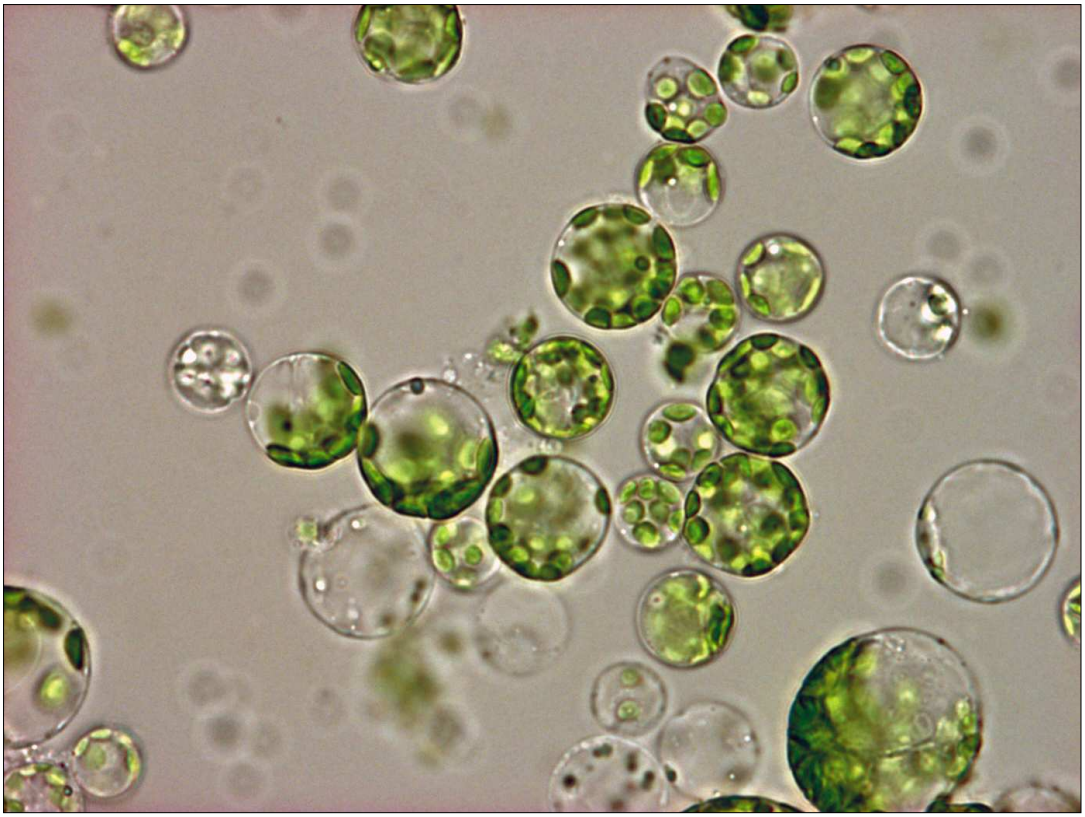


Figure 7

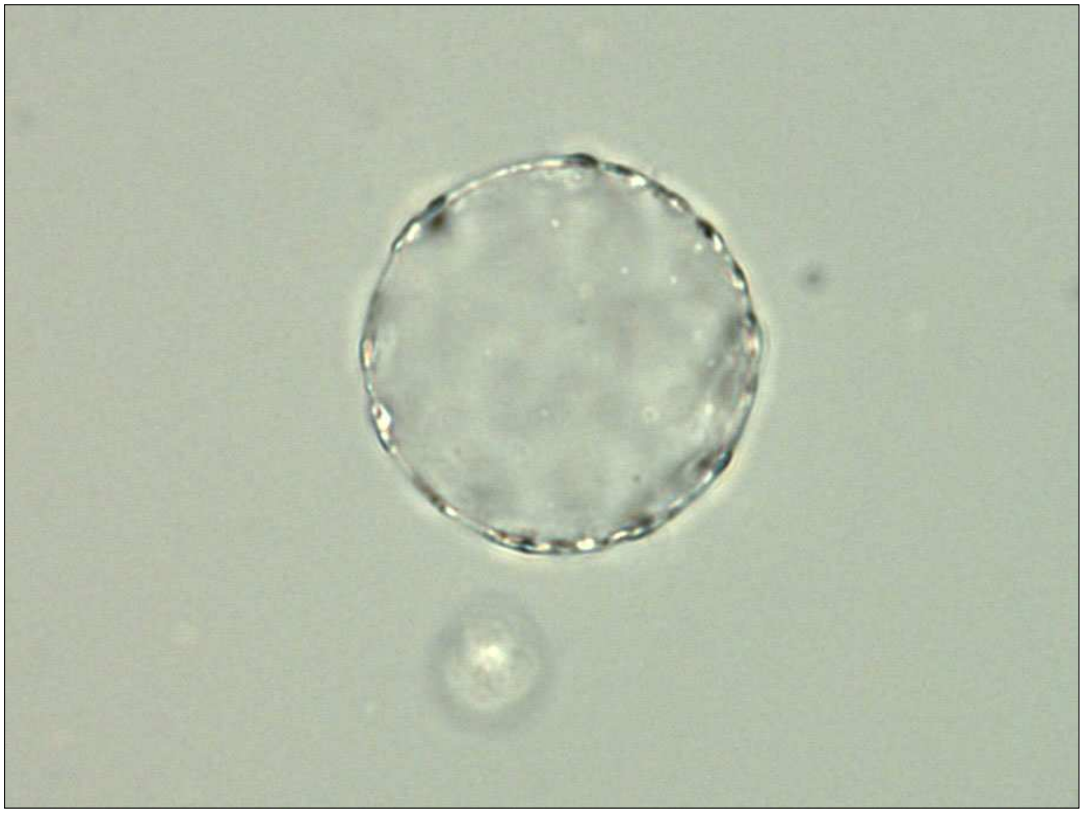


Figure 8

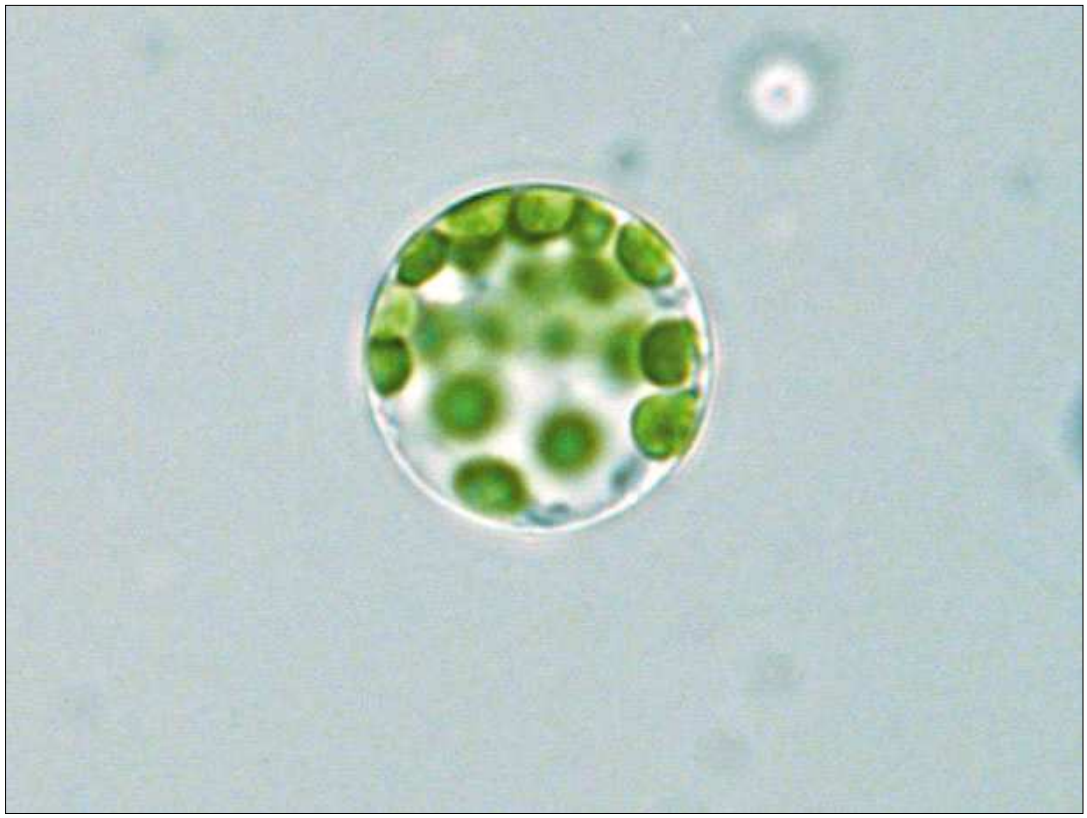


Figure 9

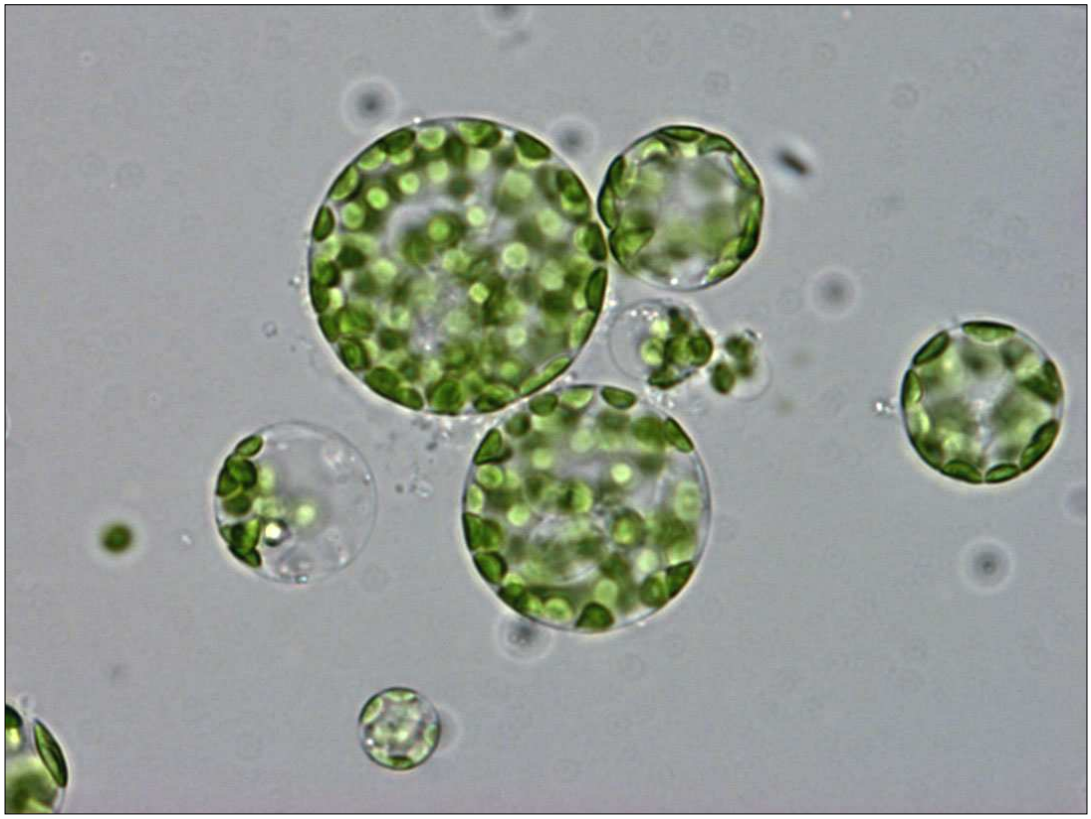


Figure 10

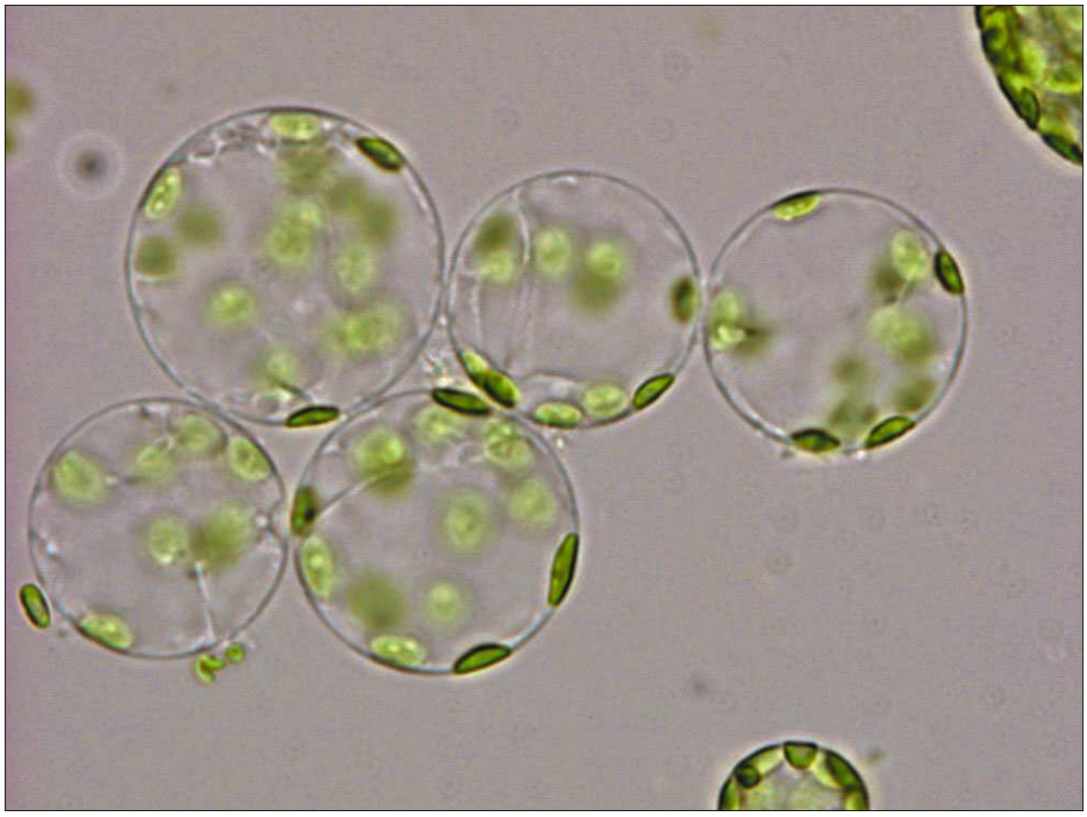


Figure 11

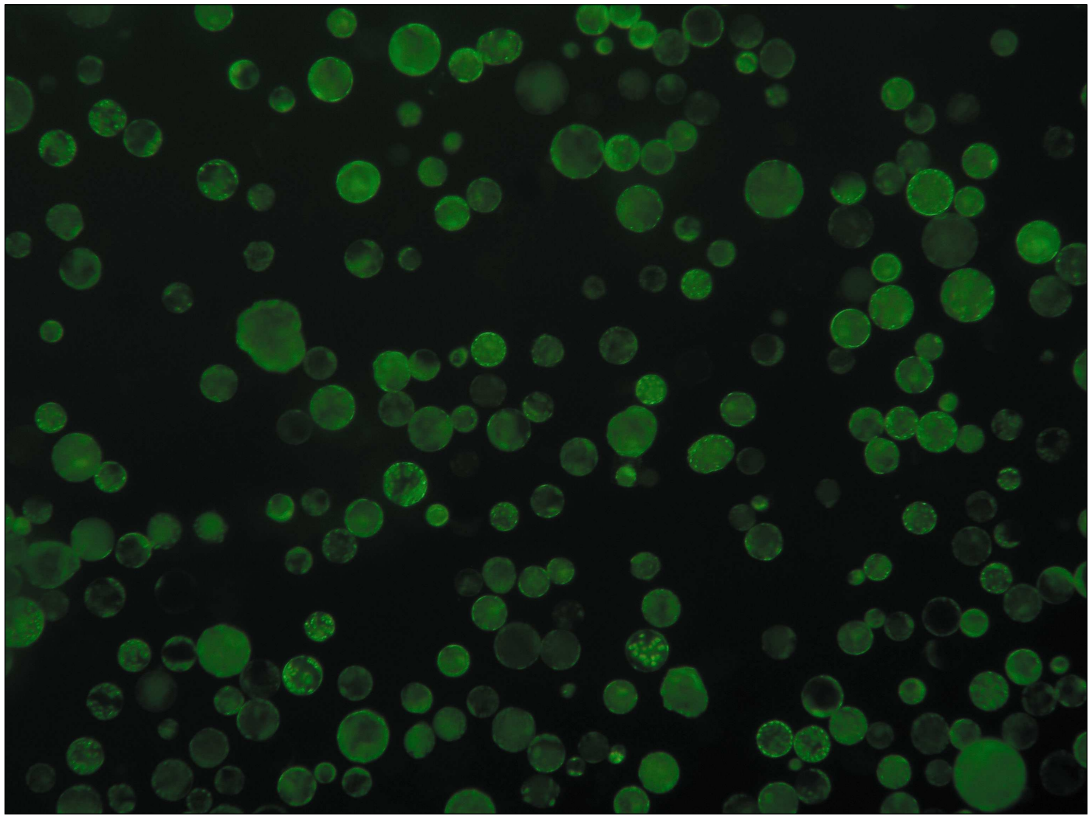


Figure 12

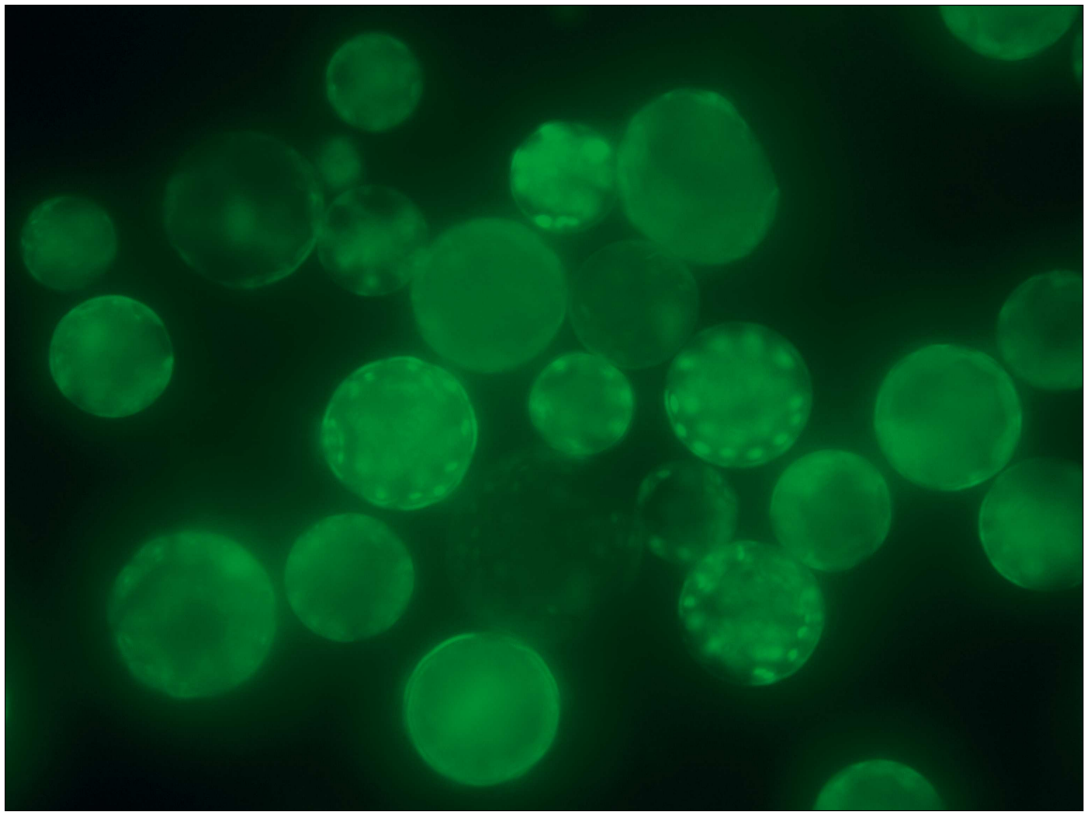


Figure 13

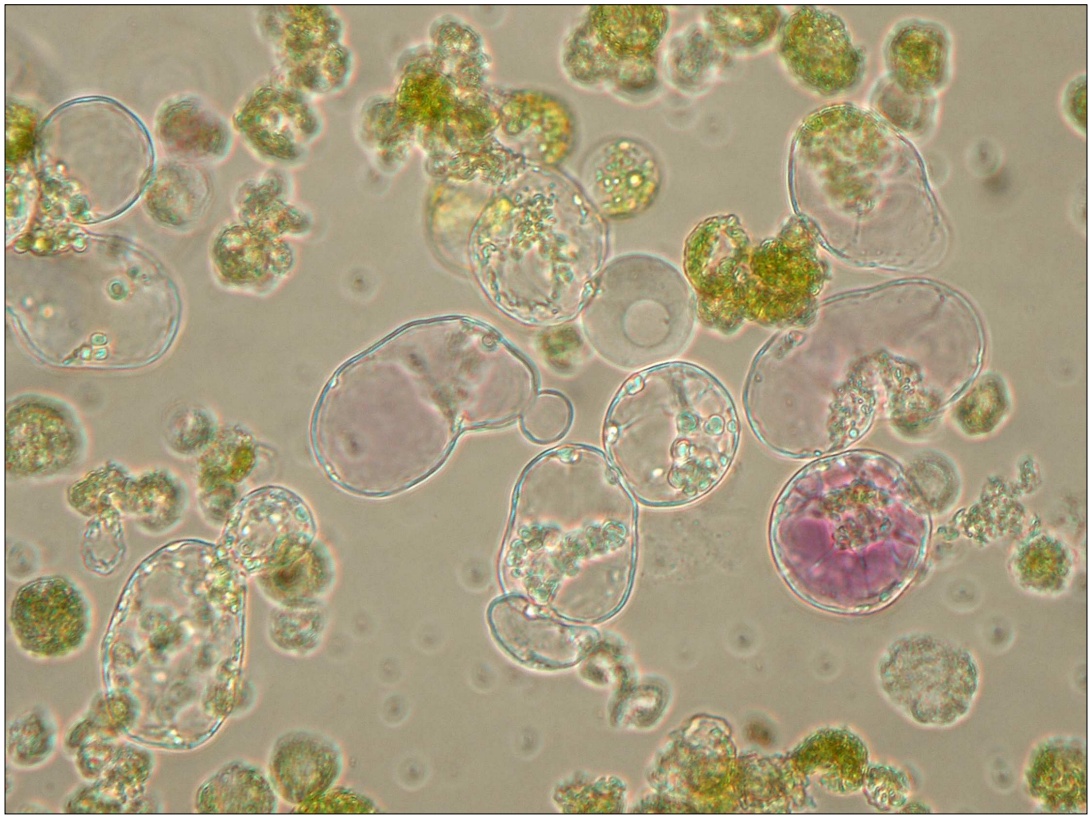


Figure 14

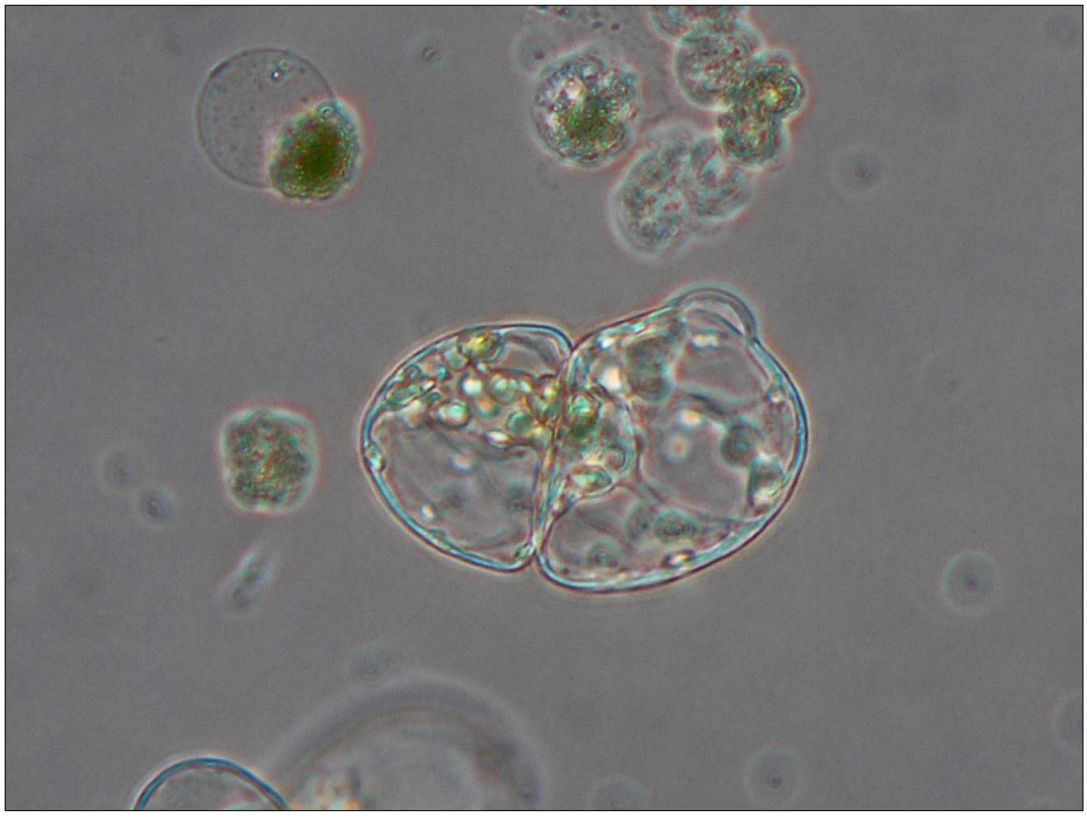


Figure 15

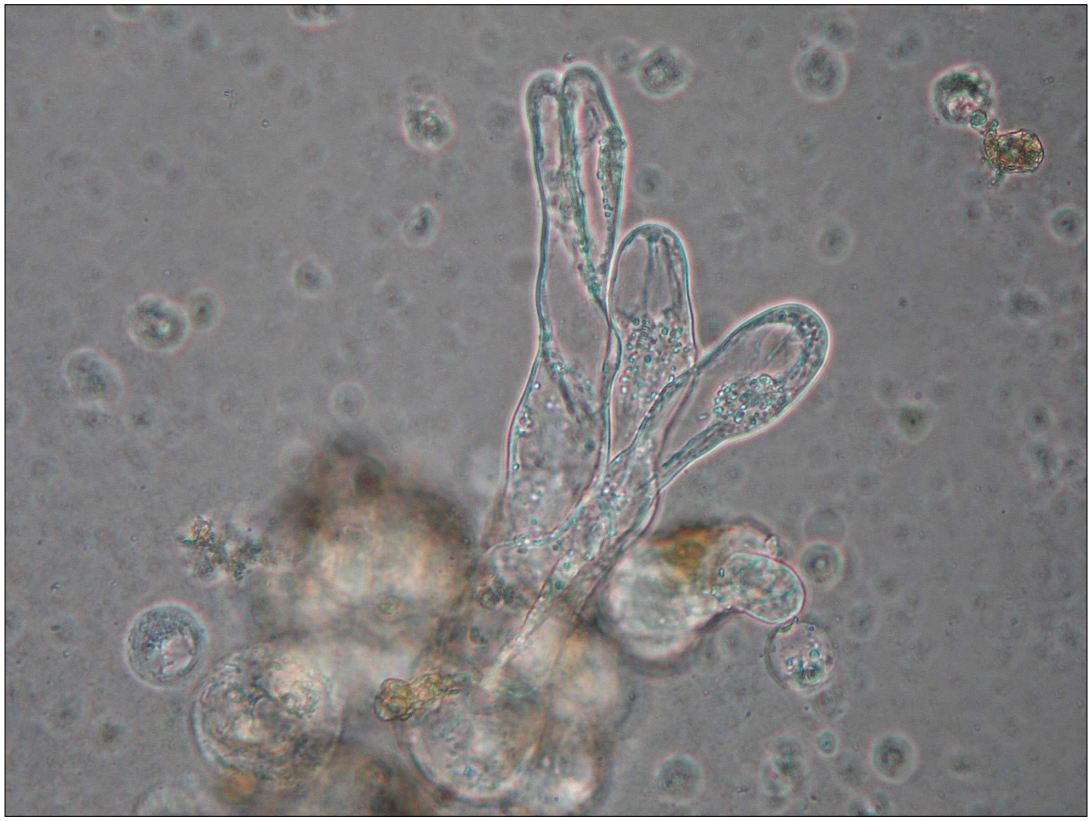
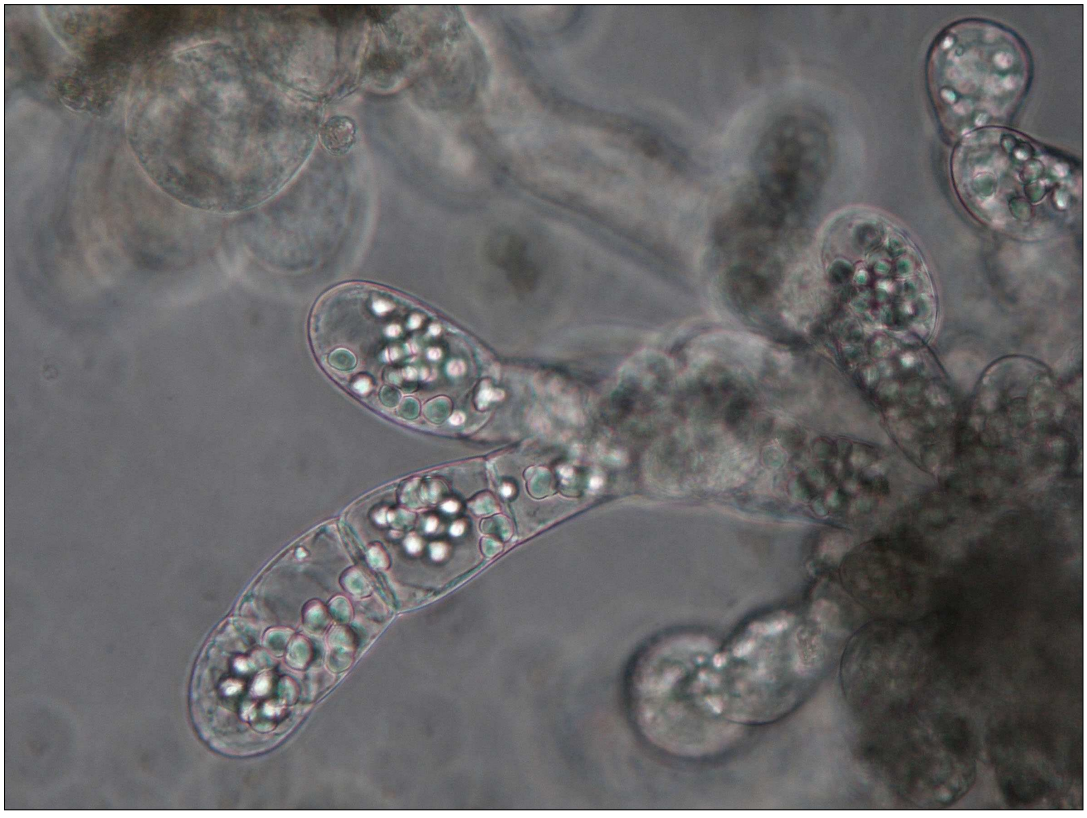


Figure 16



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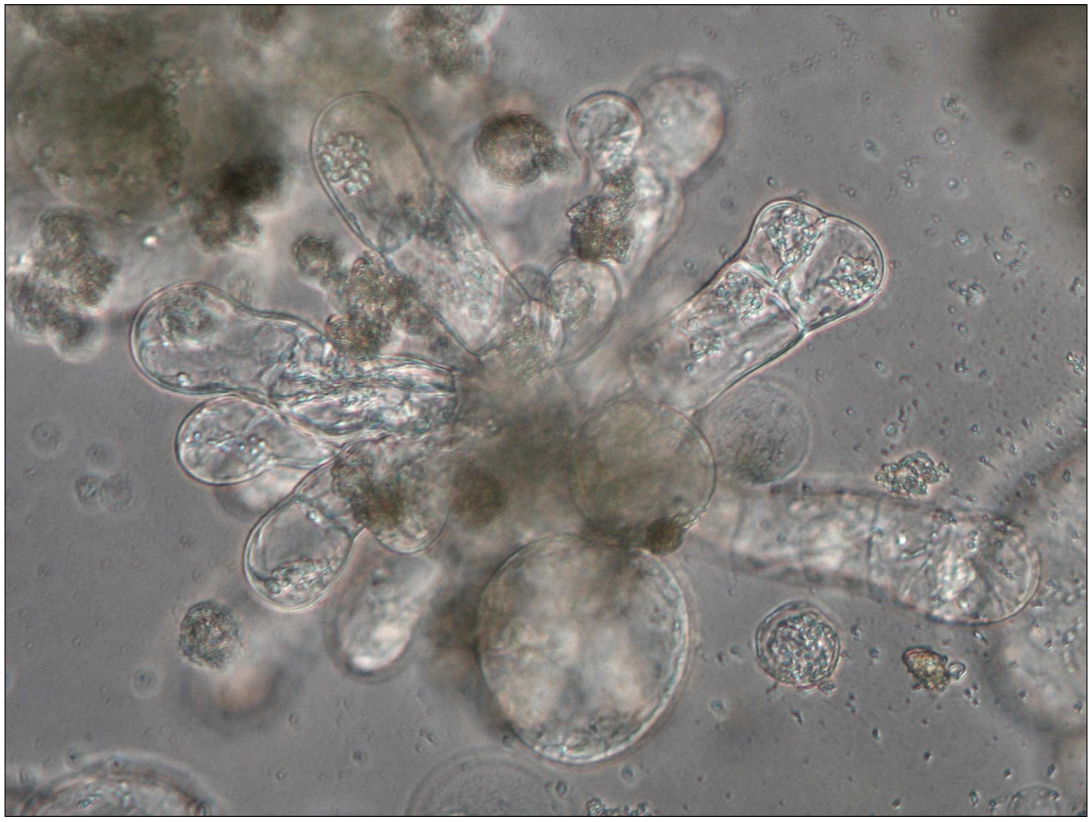

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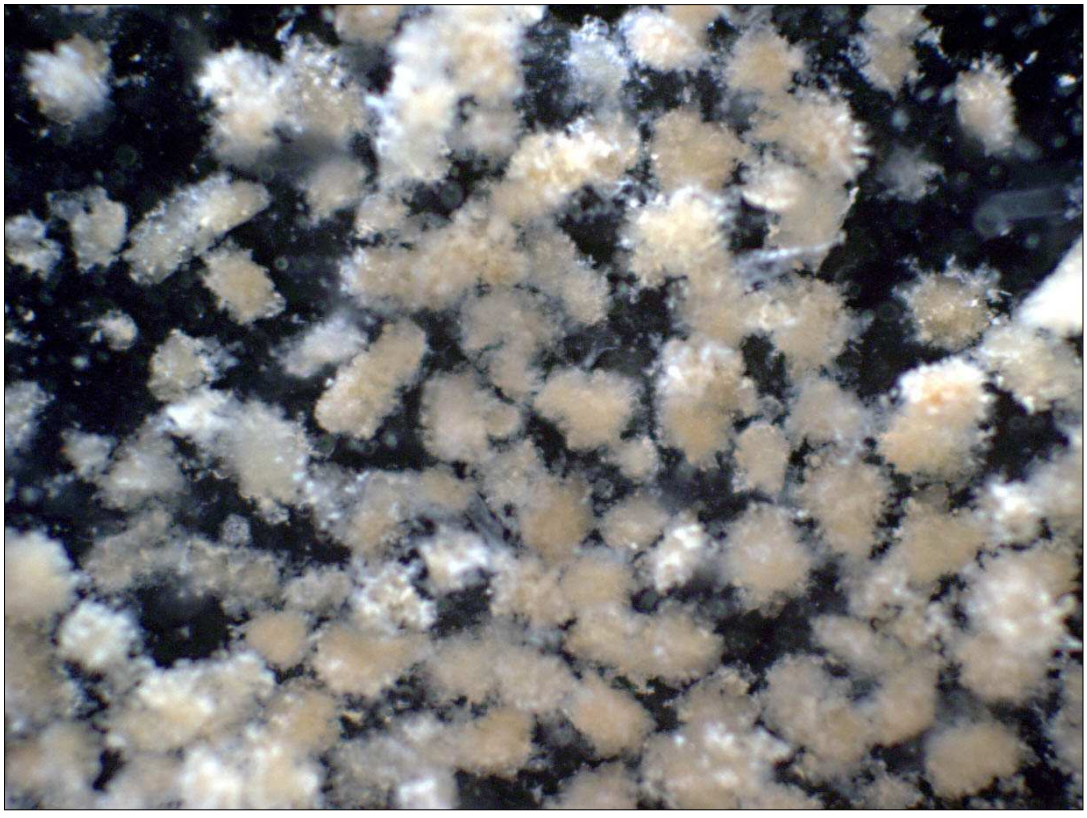

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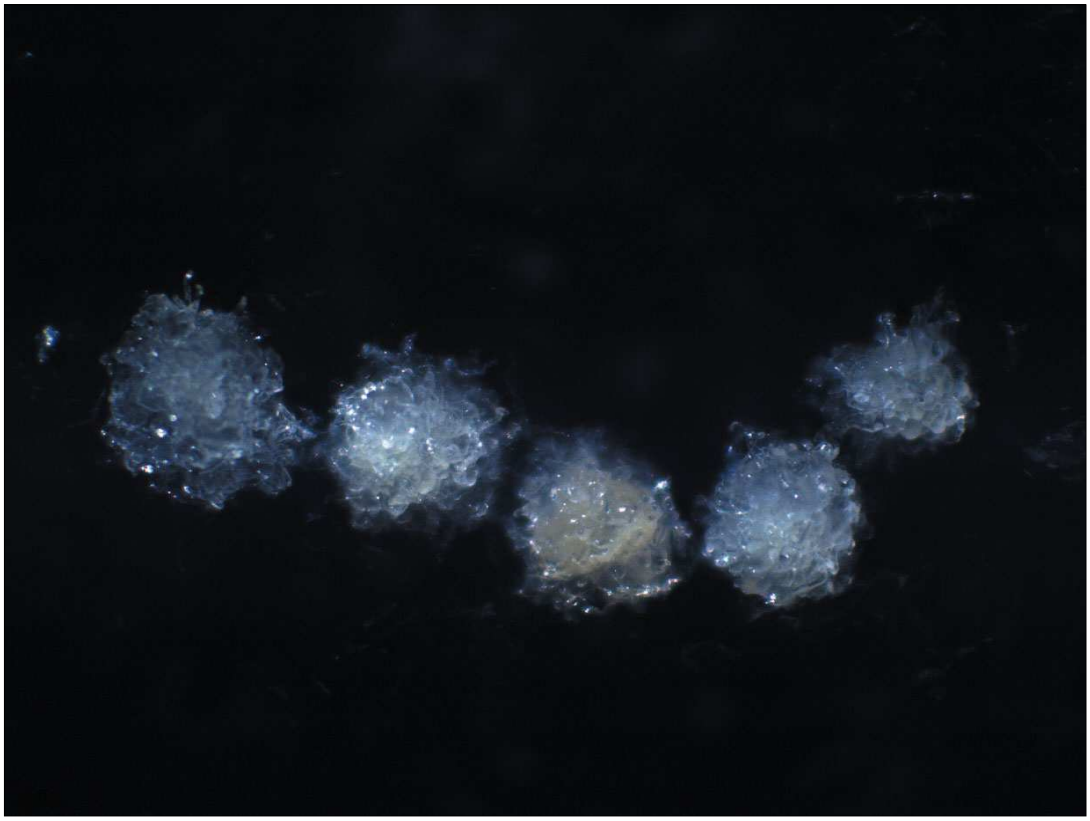

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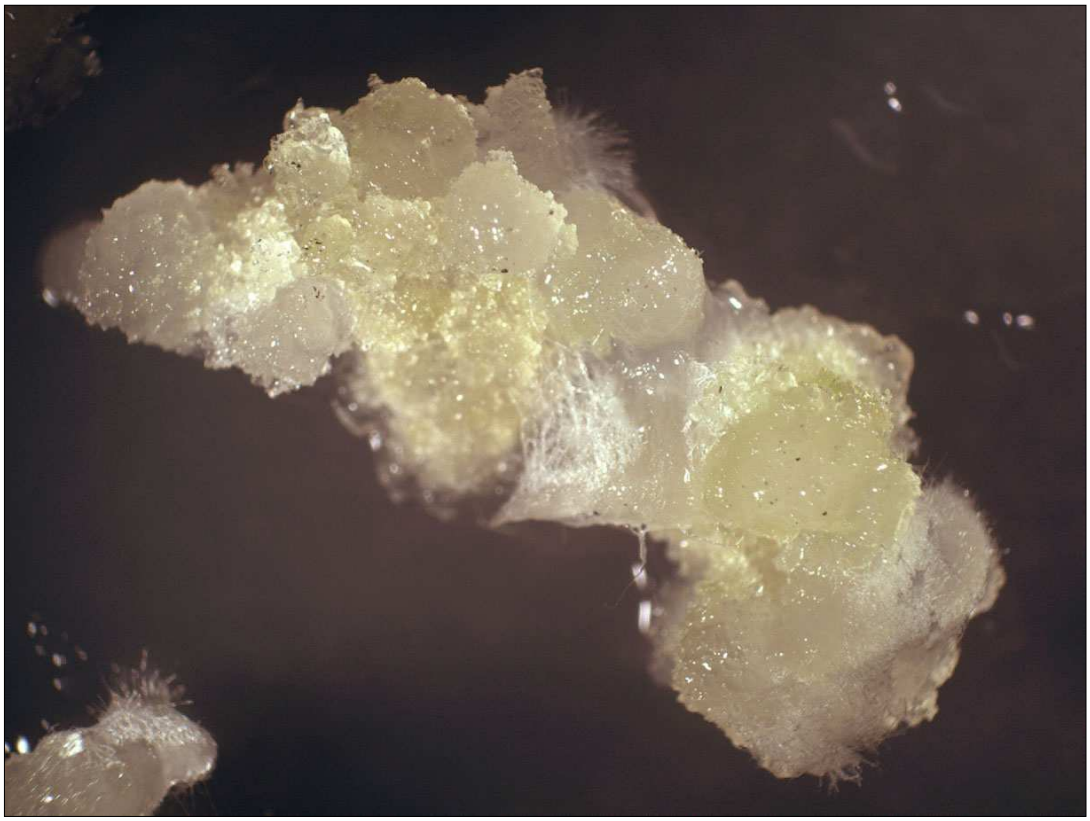

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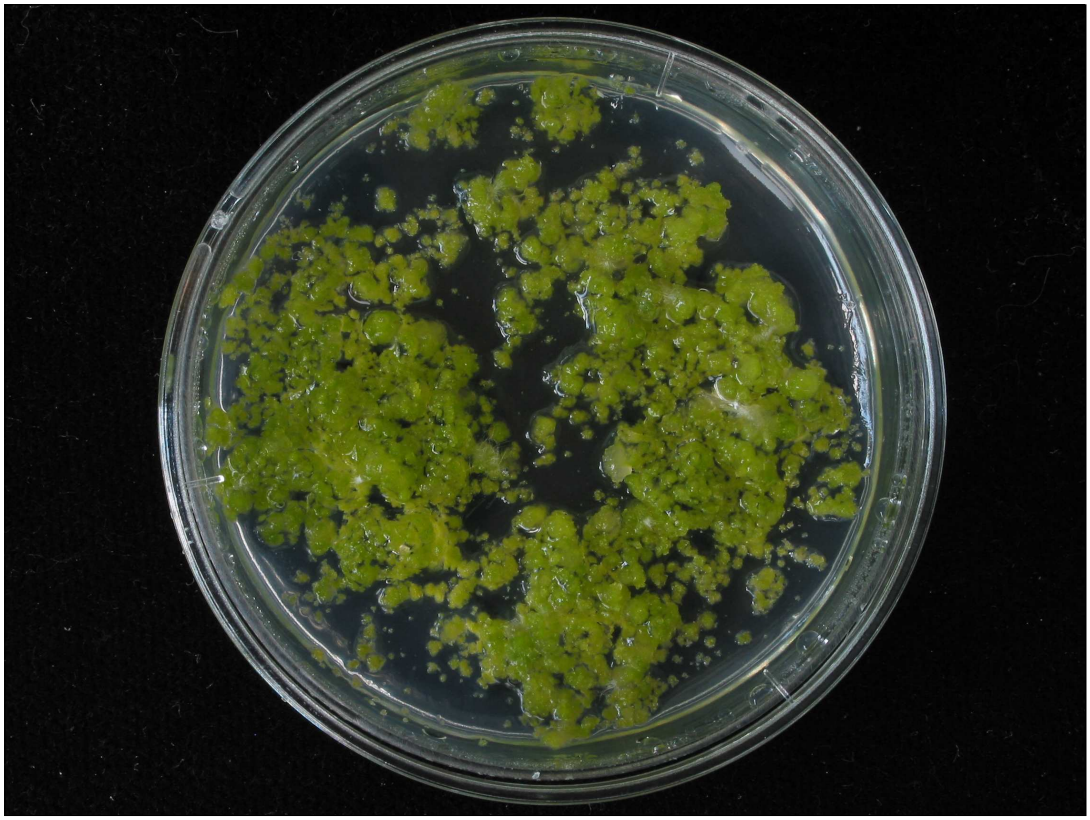

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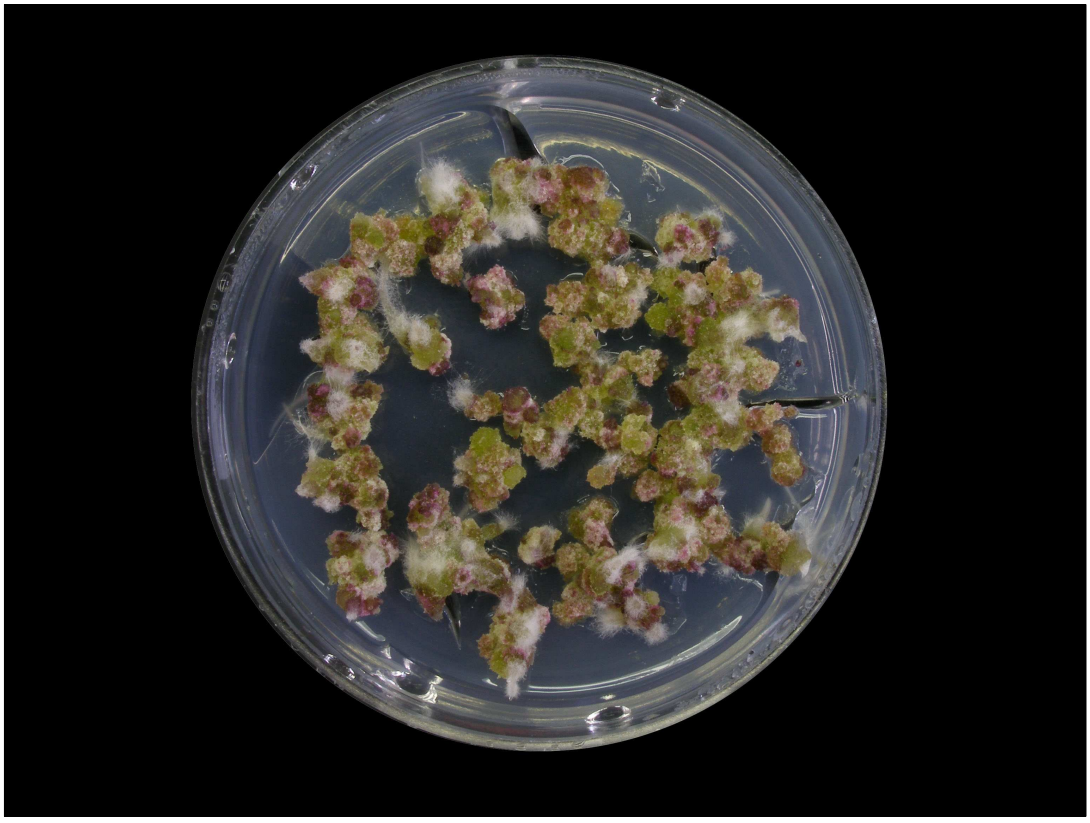
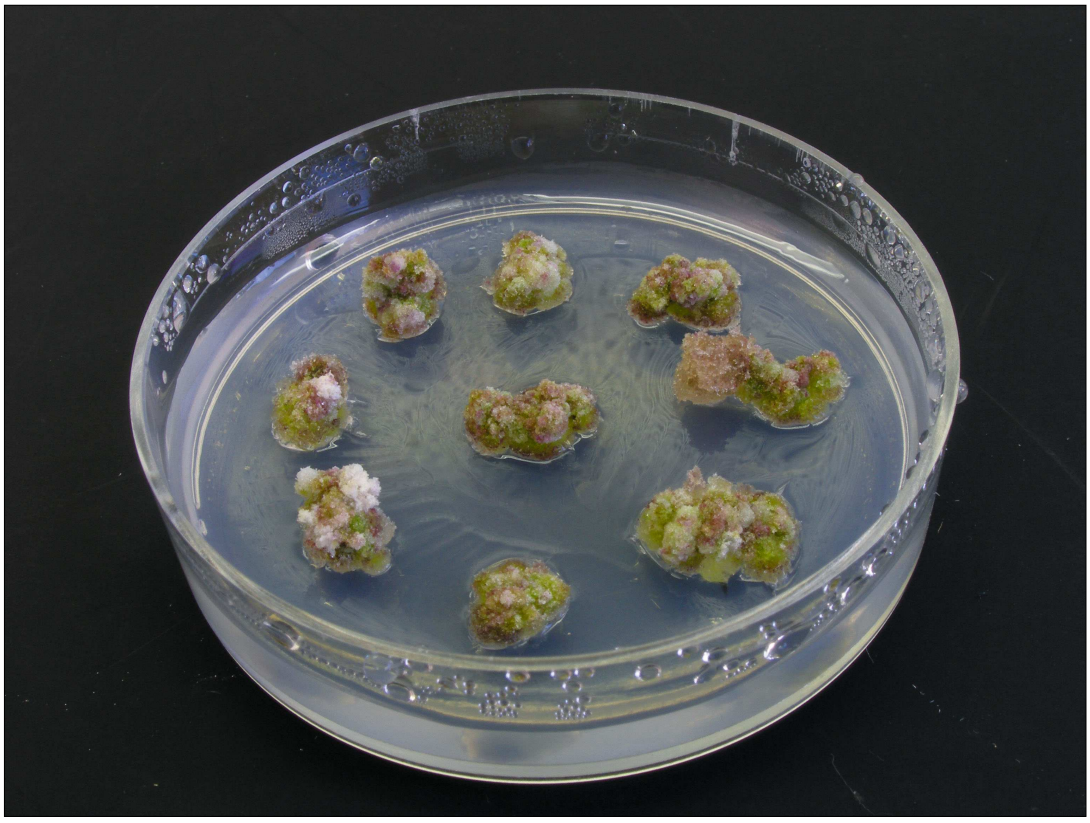


Figure 23

Figure 24



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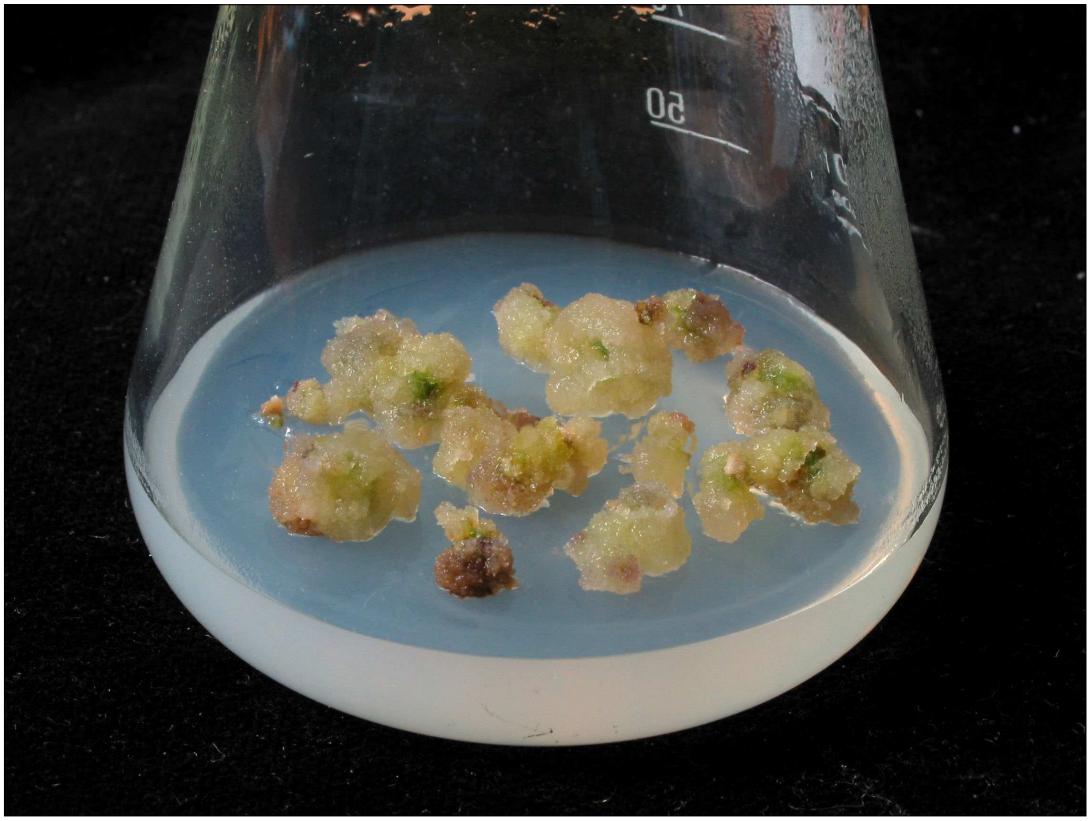

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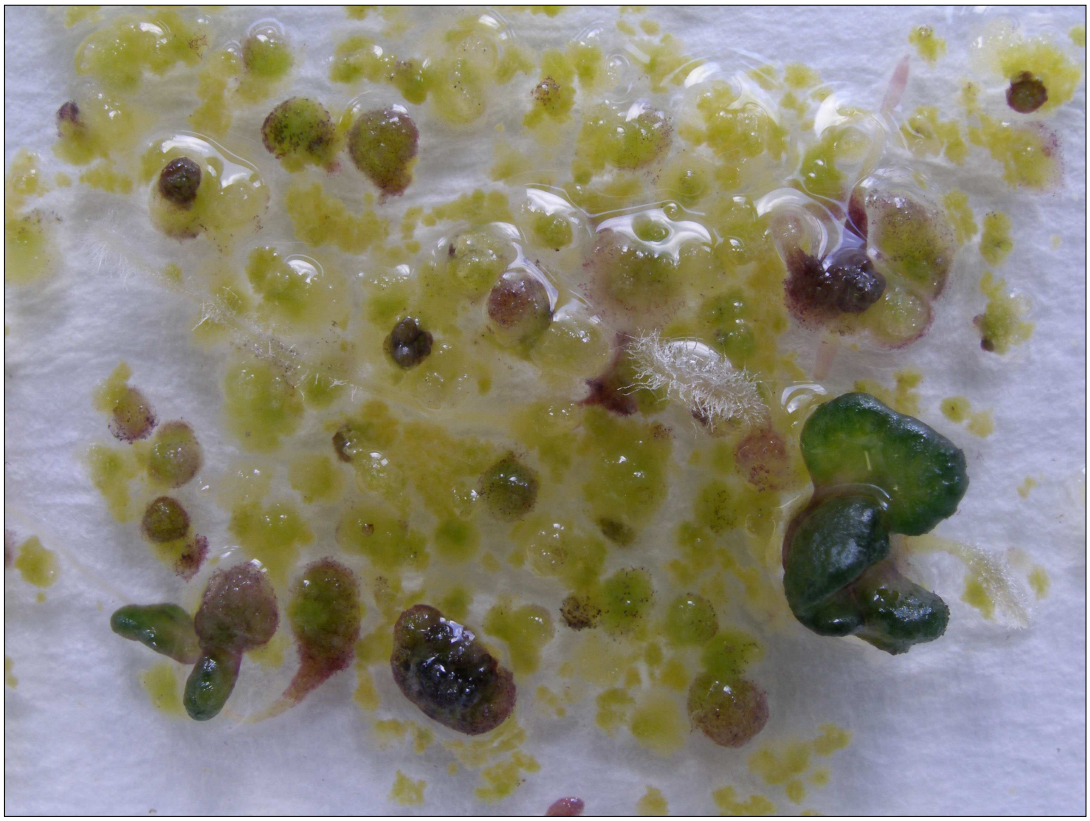

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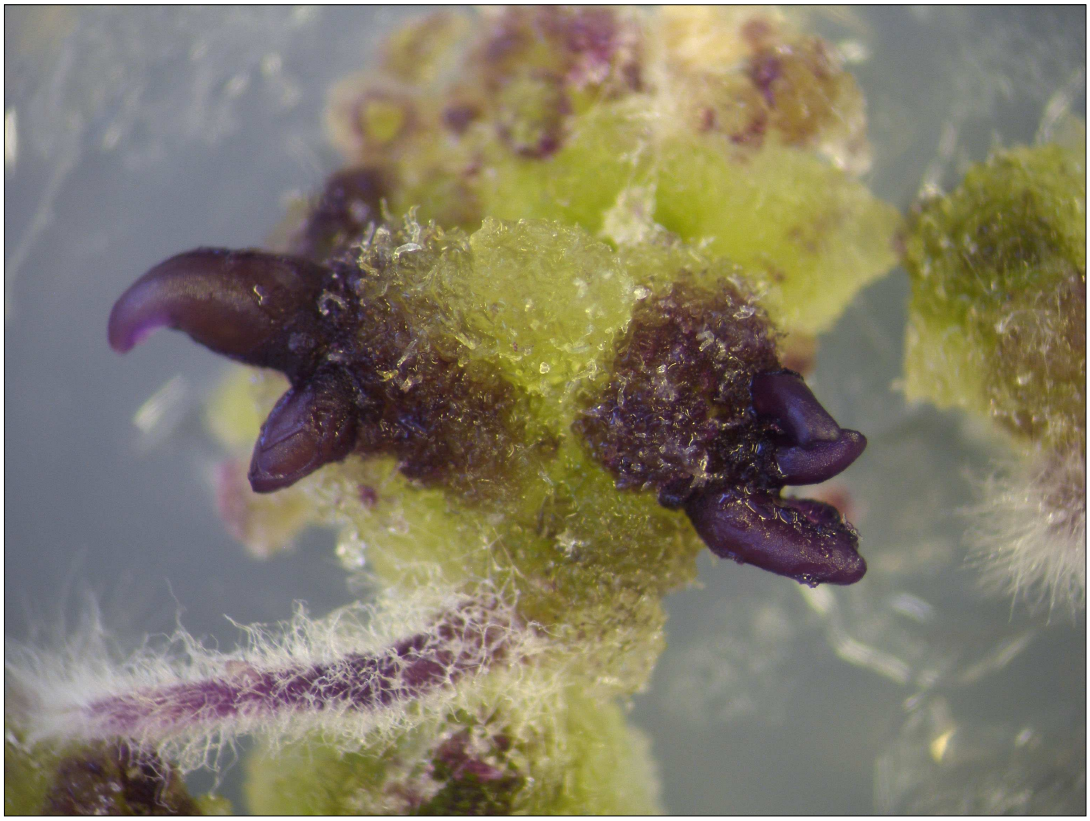


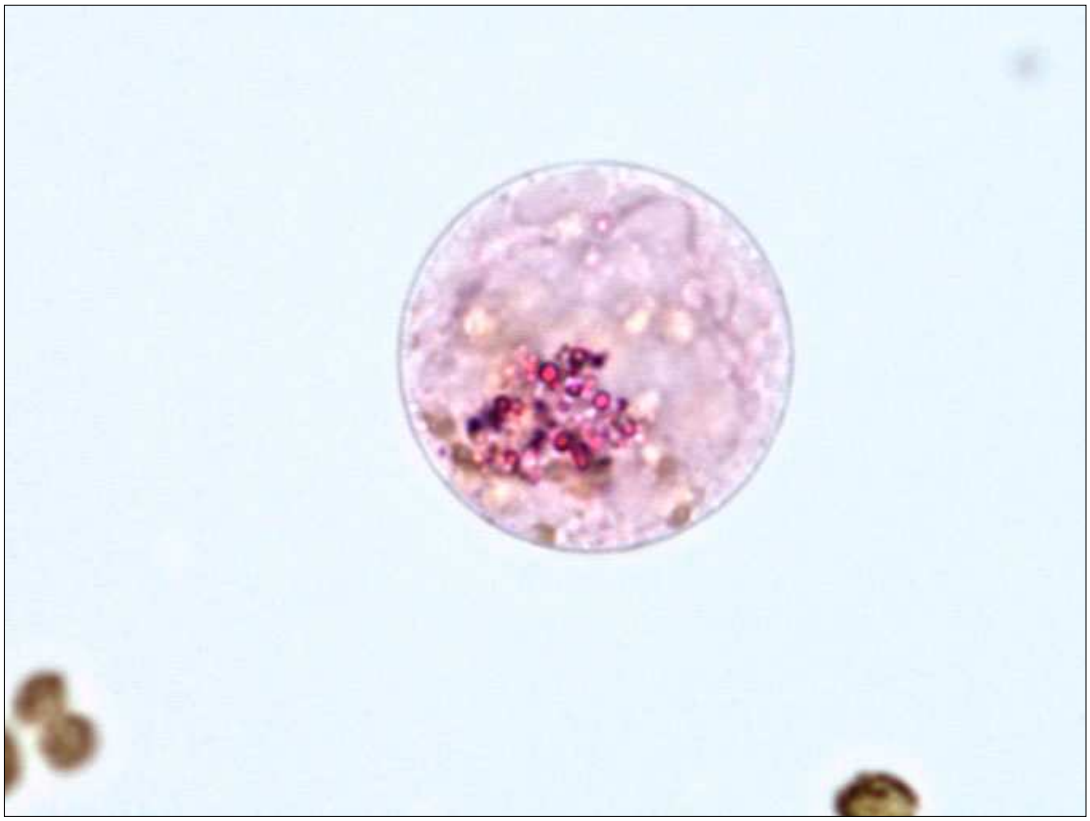
Figure 28

Figure 29

Figure 30

Figure 31

Figure 32



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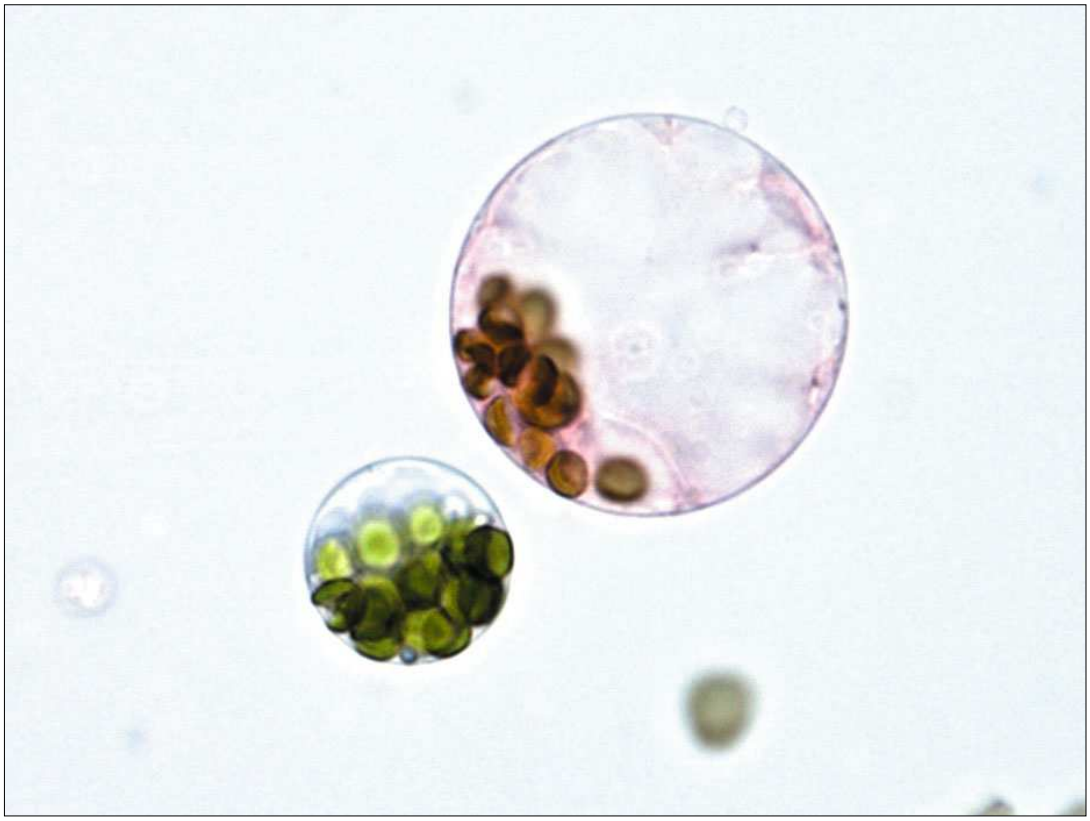

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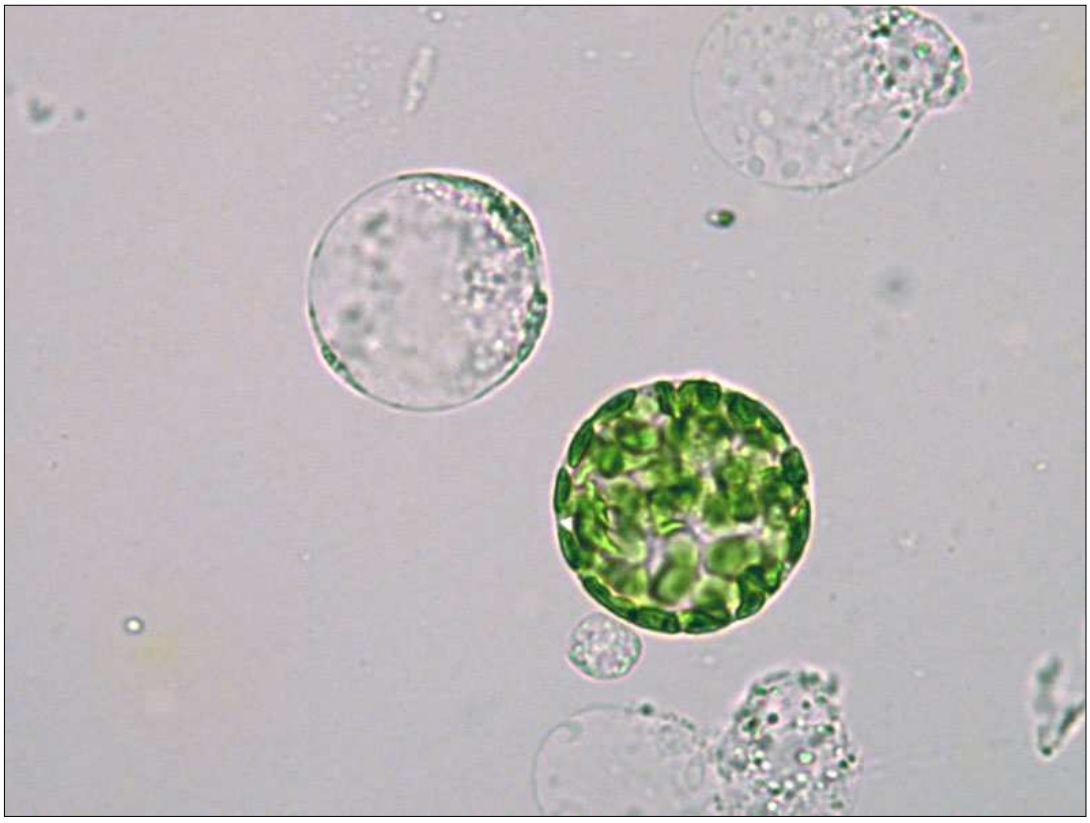

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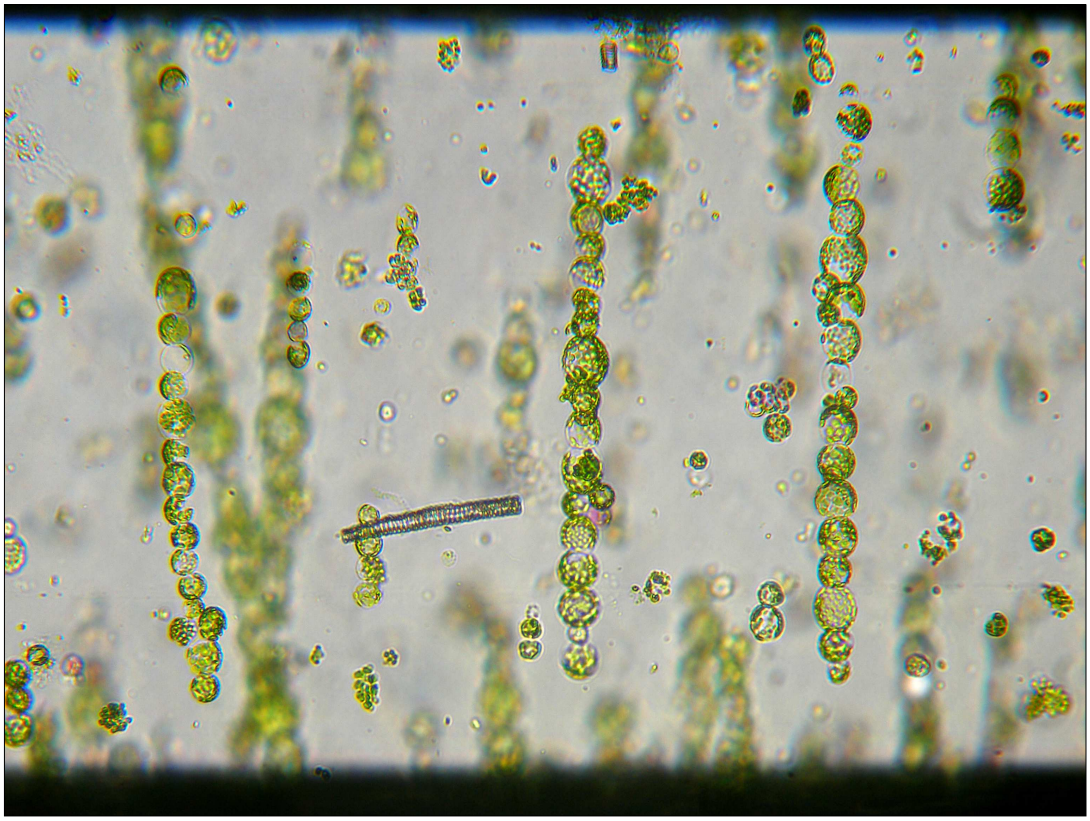

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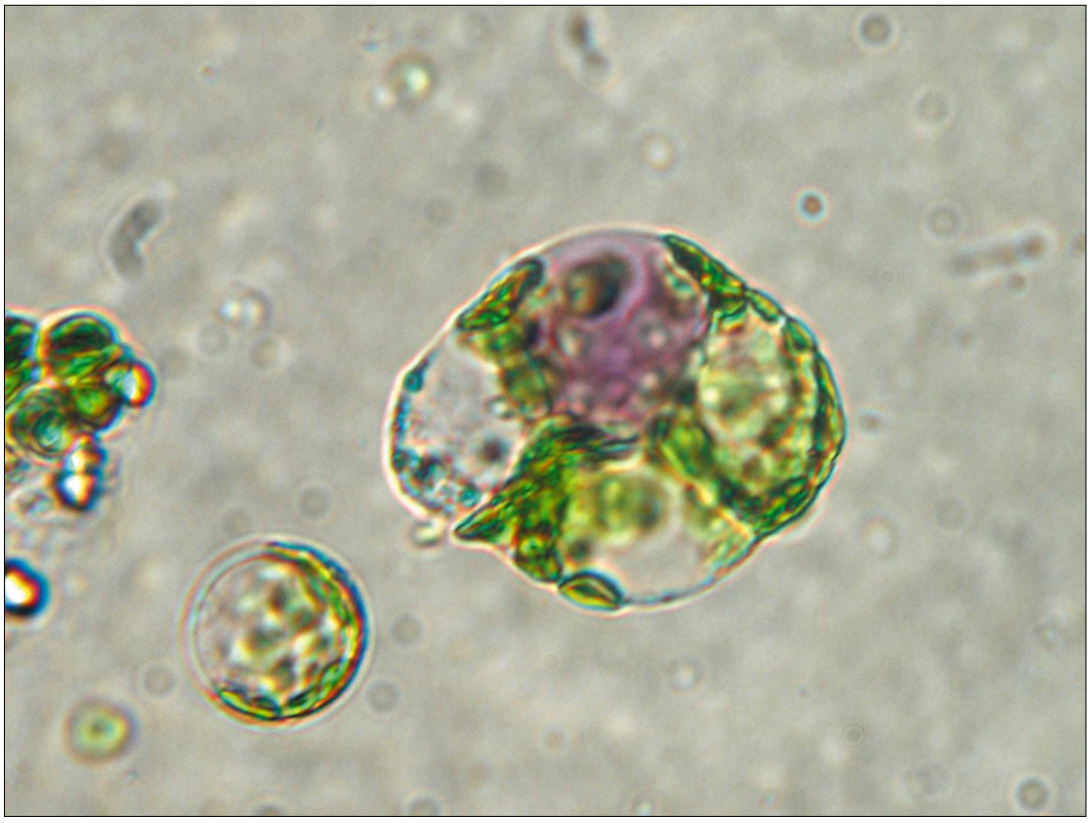

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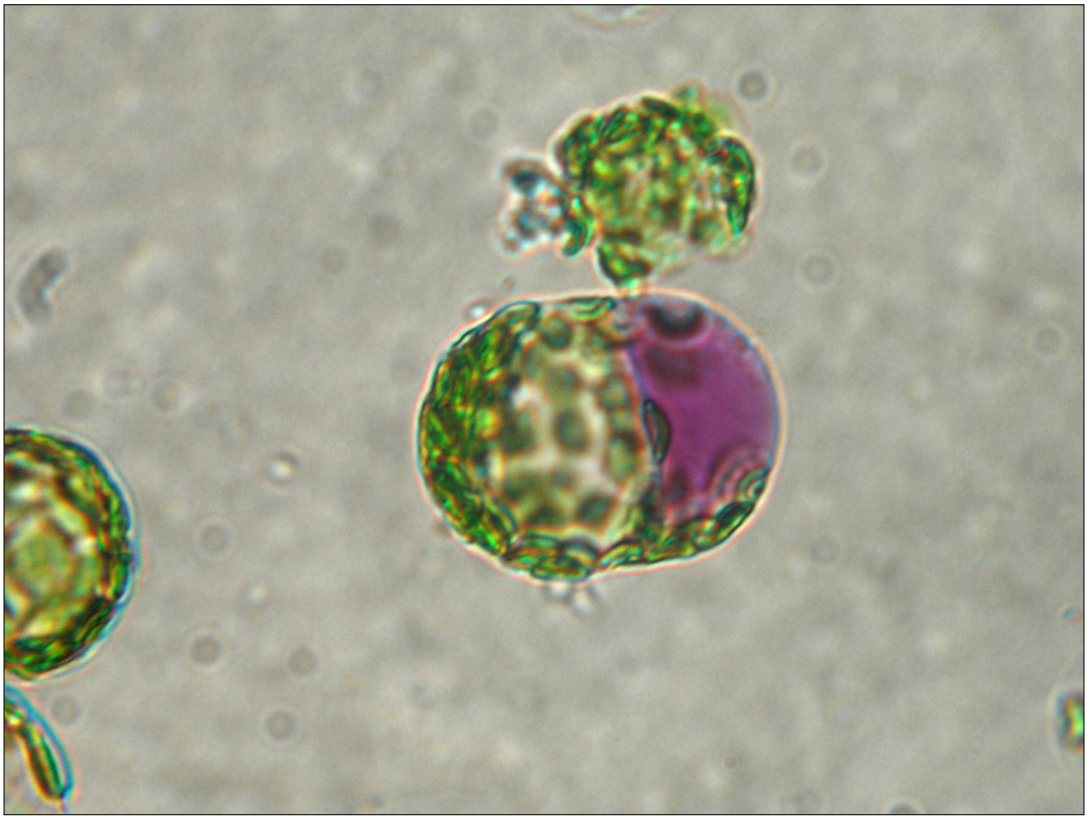

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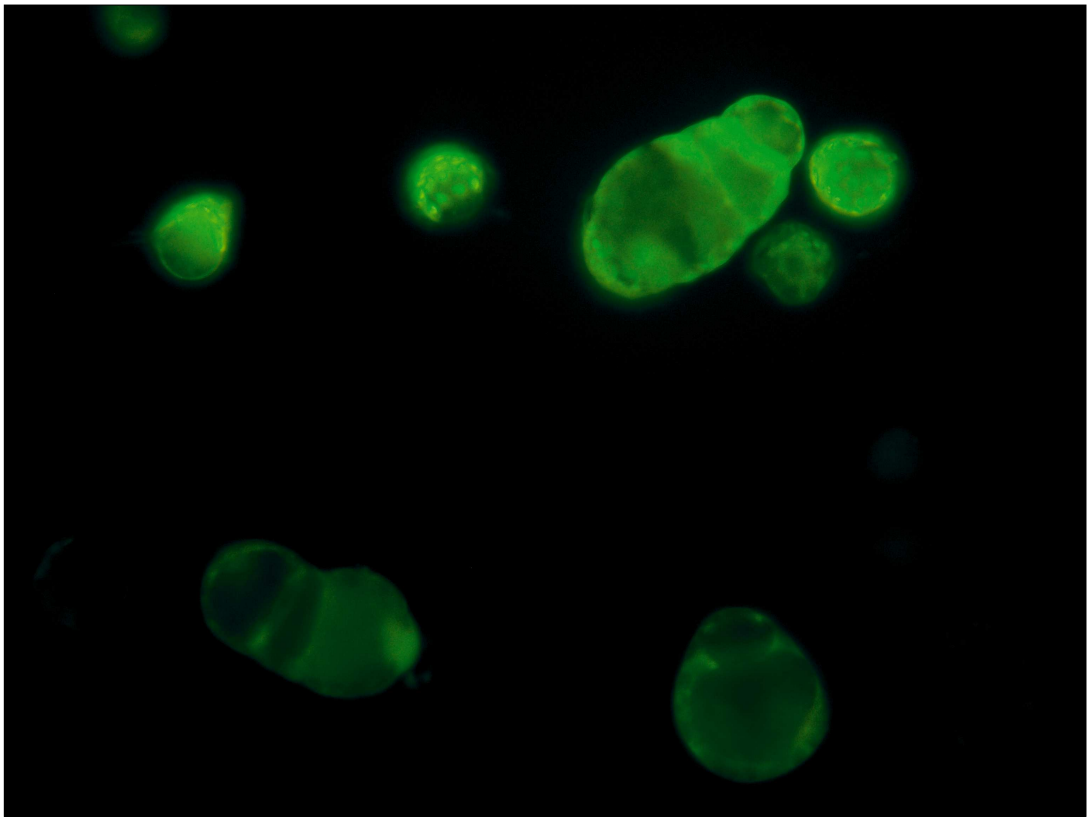

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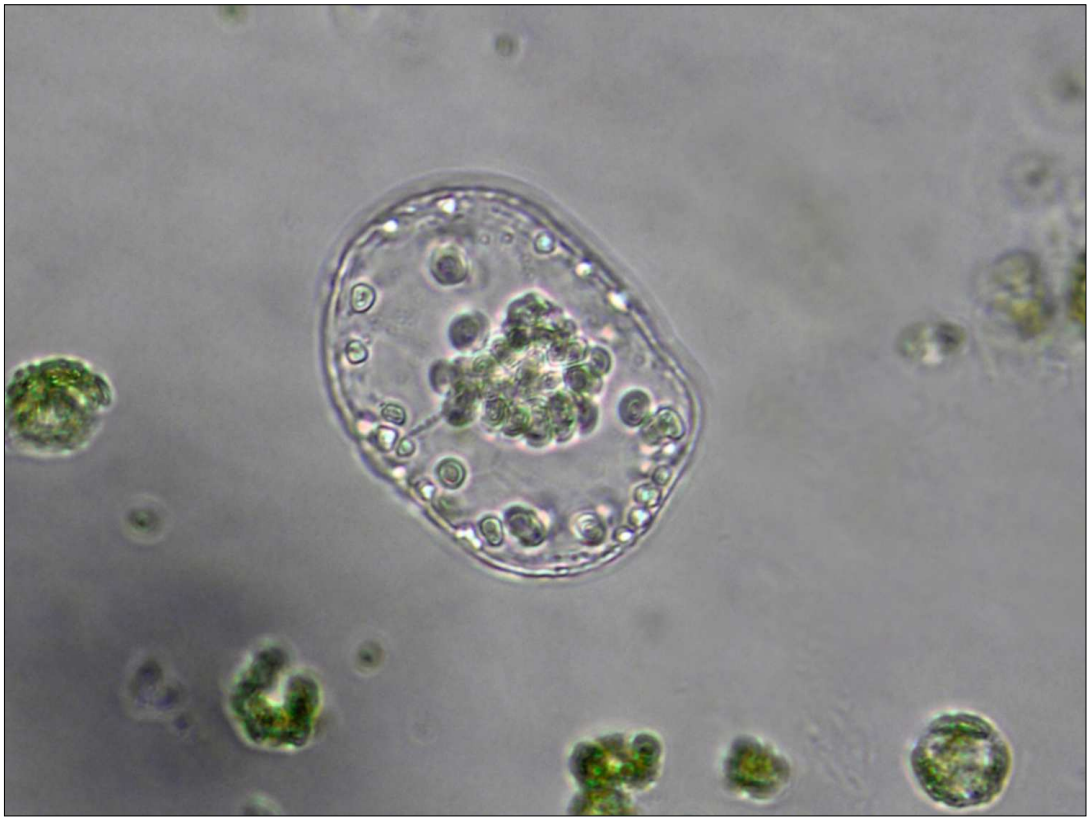

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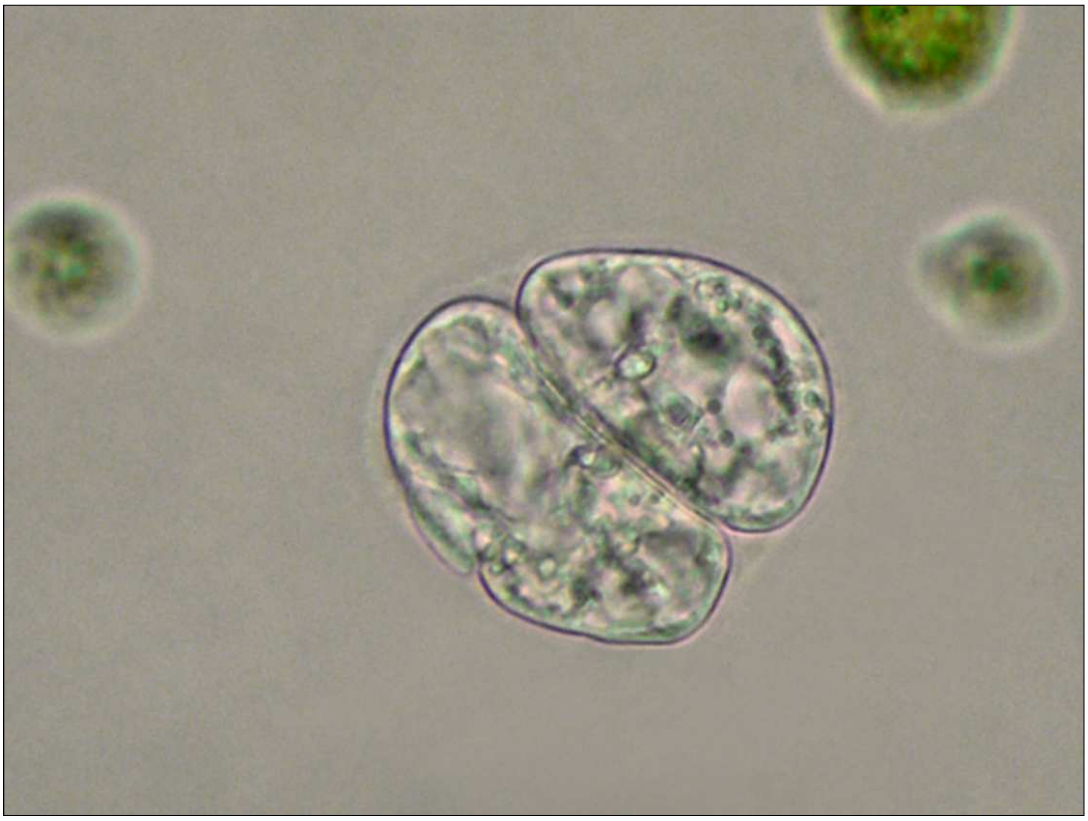

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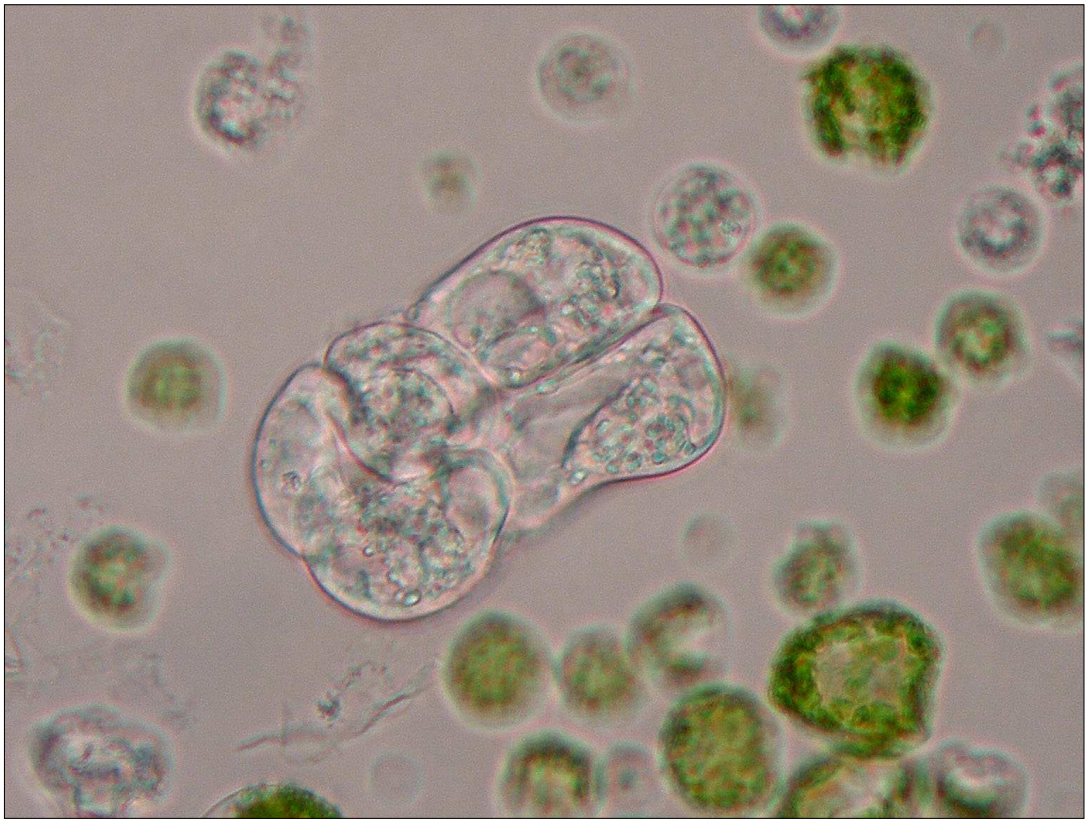

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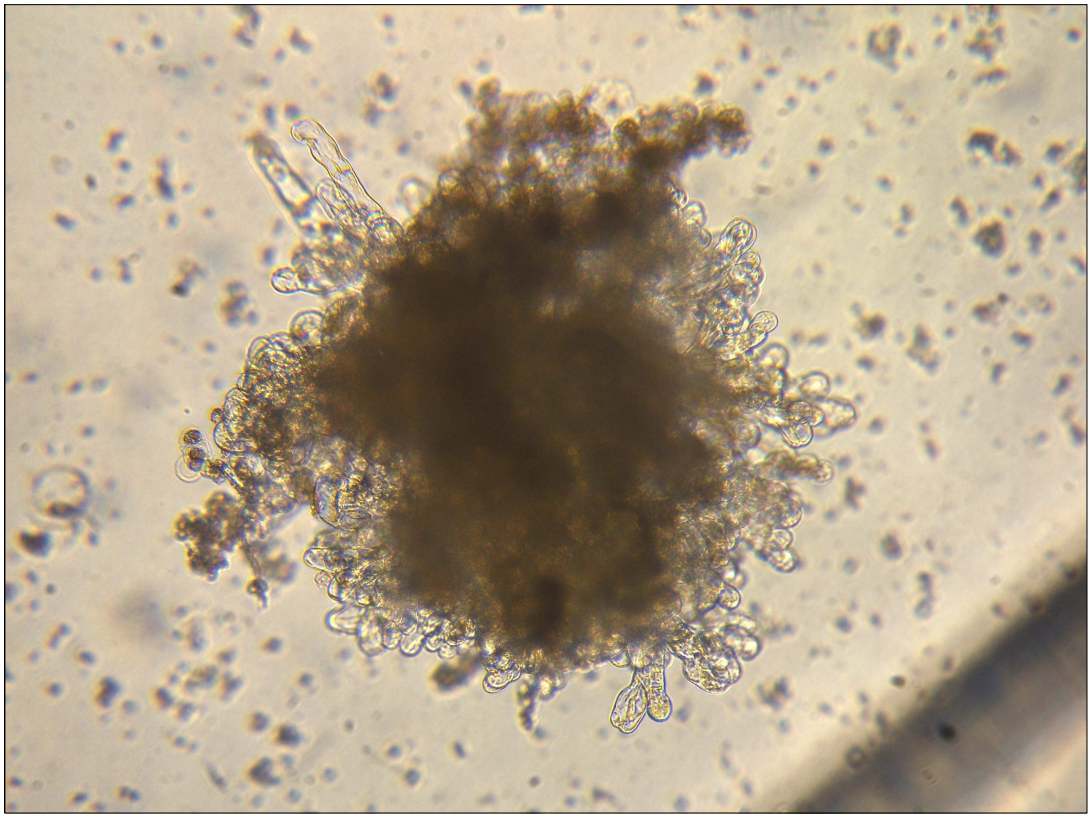

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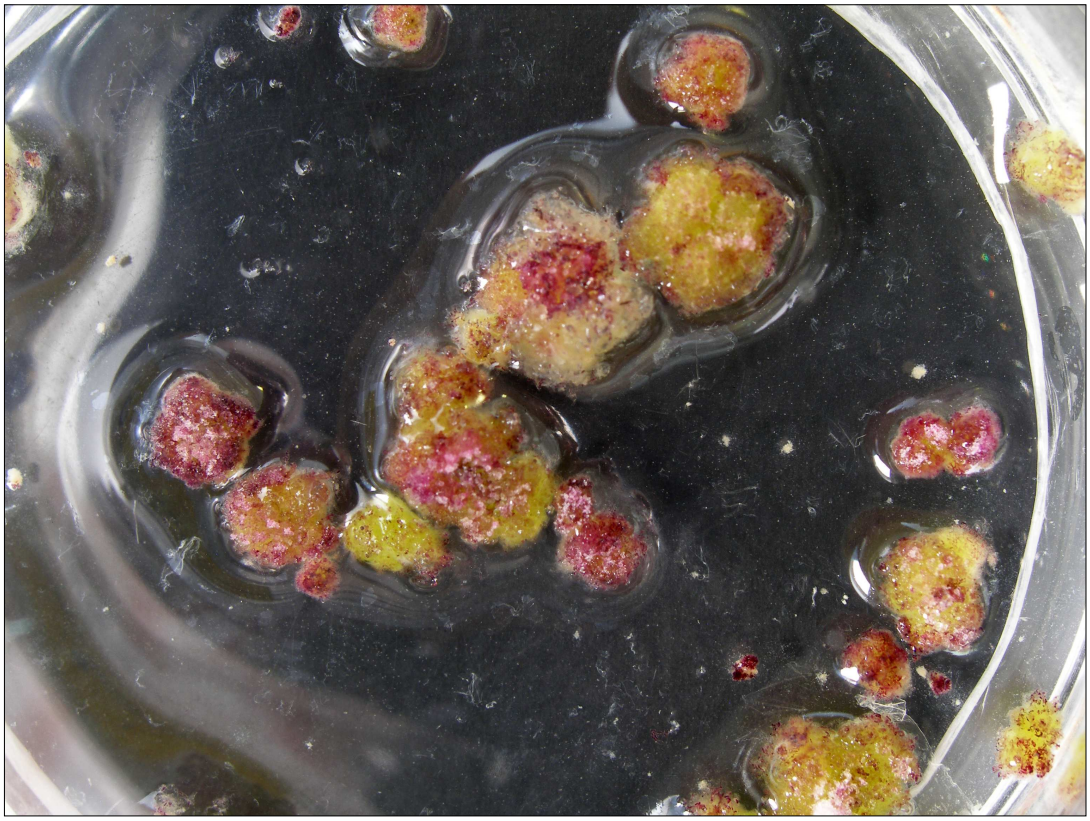

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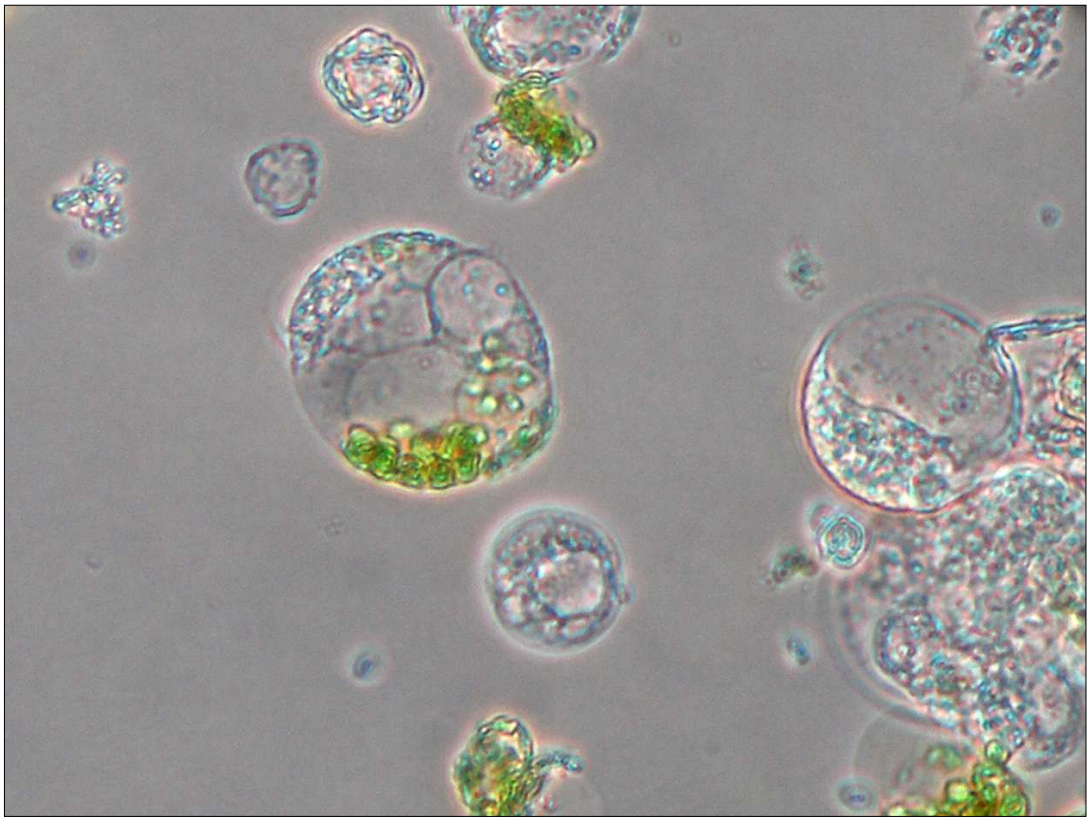

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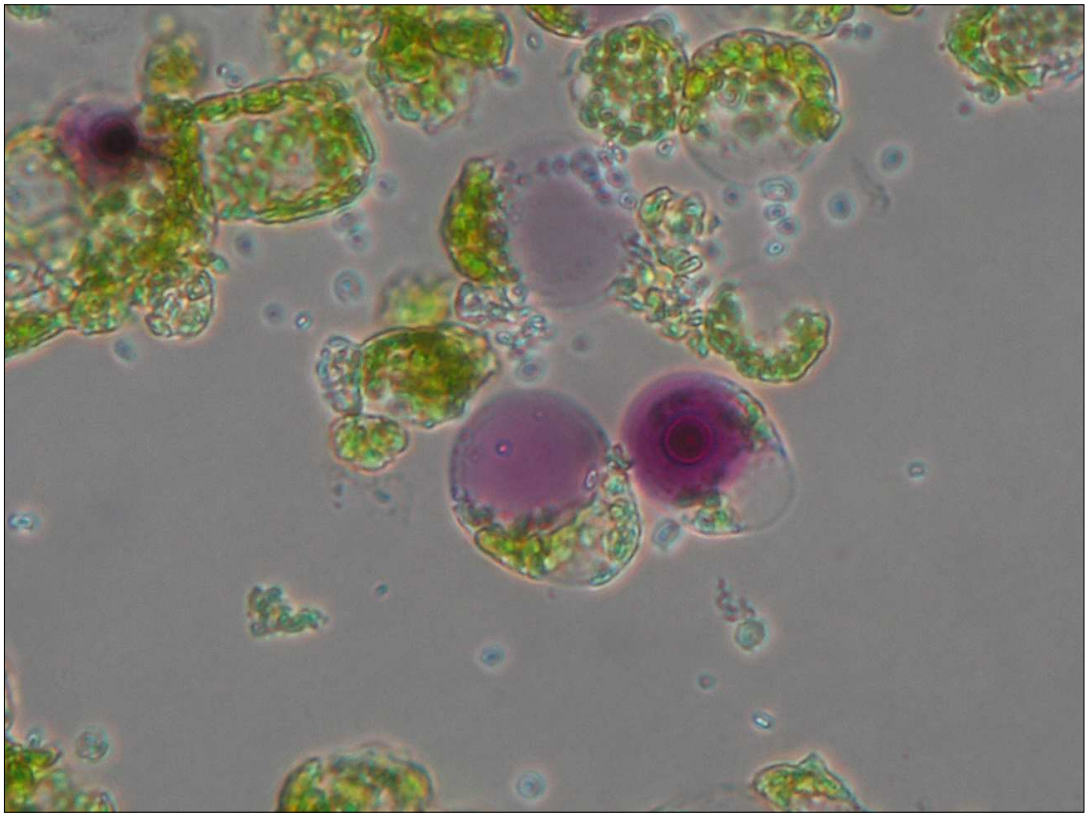

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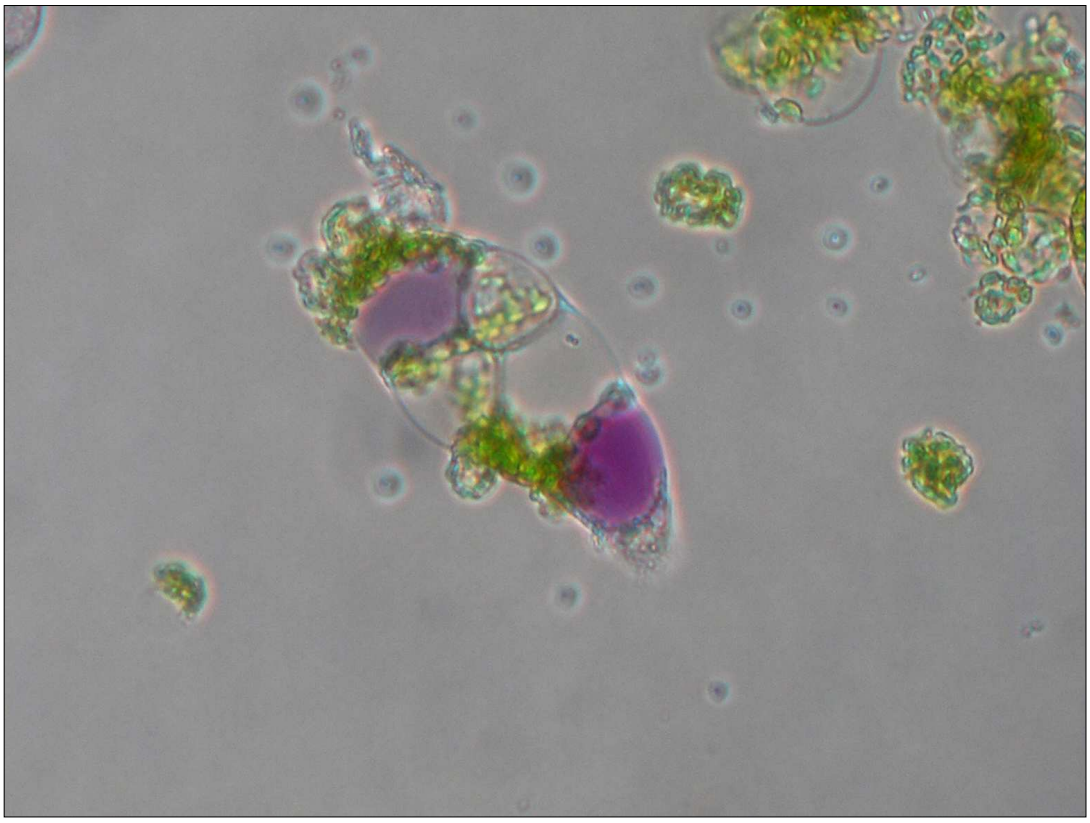

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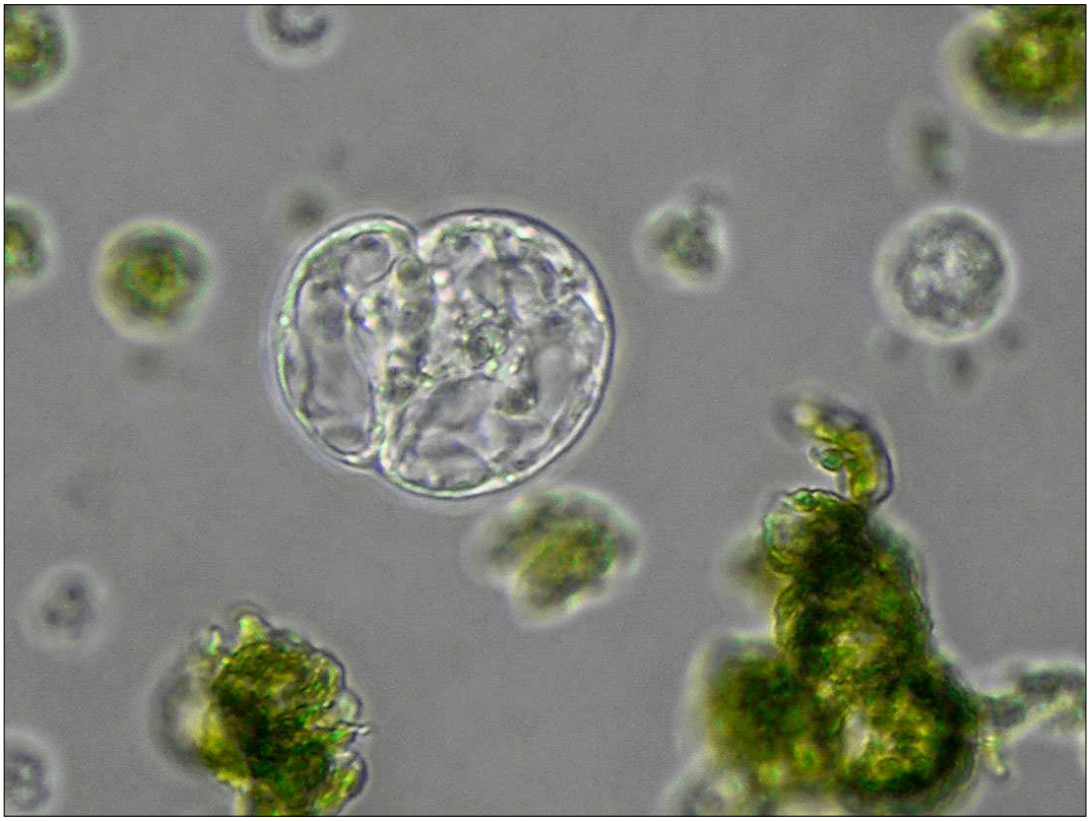

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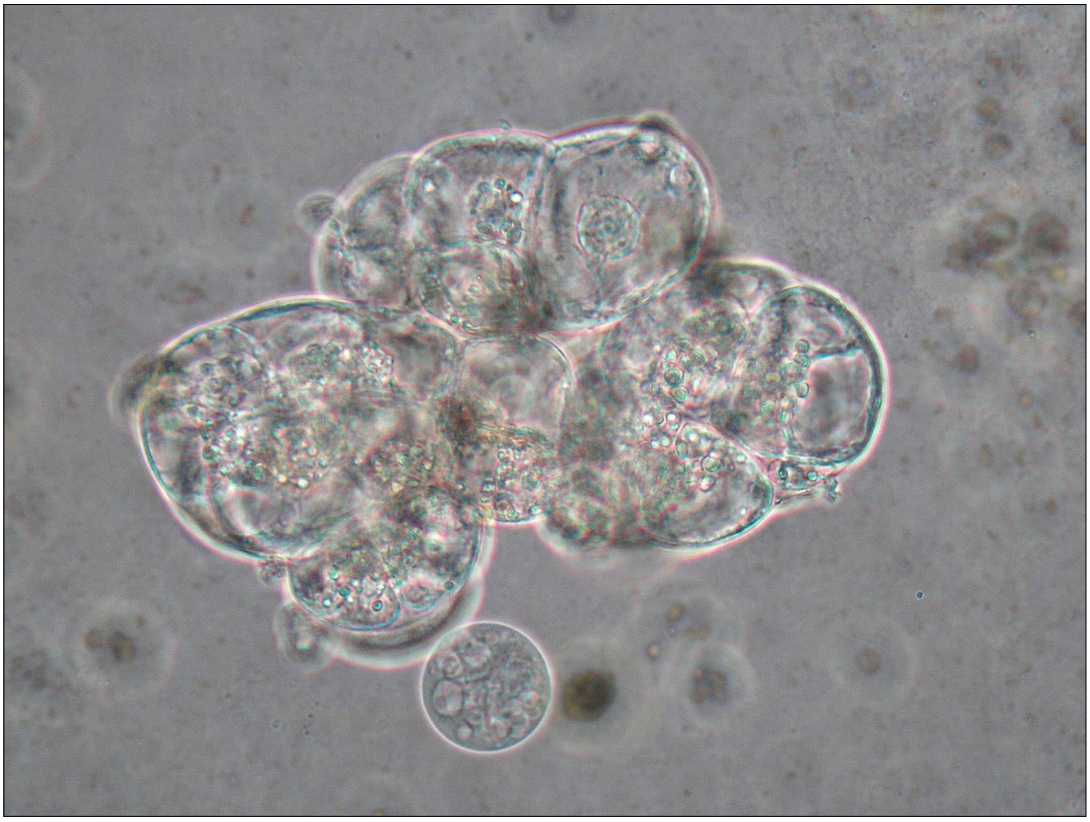

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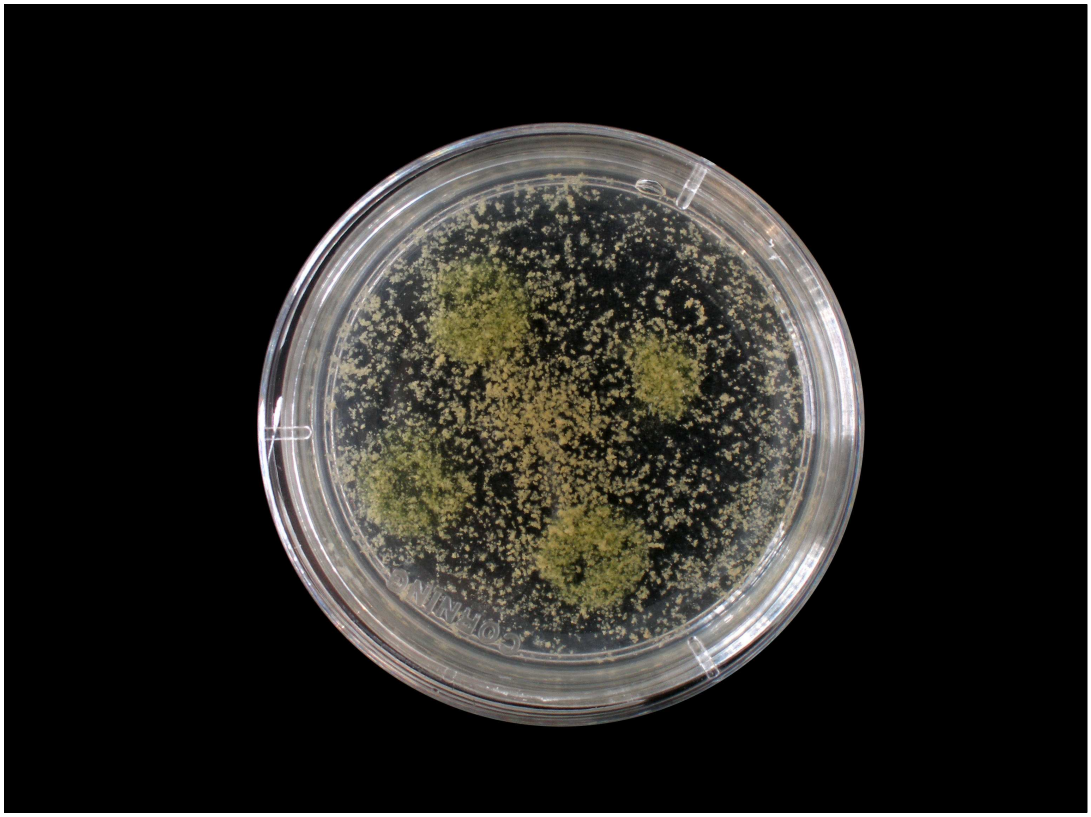

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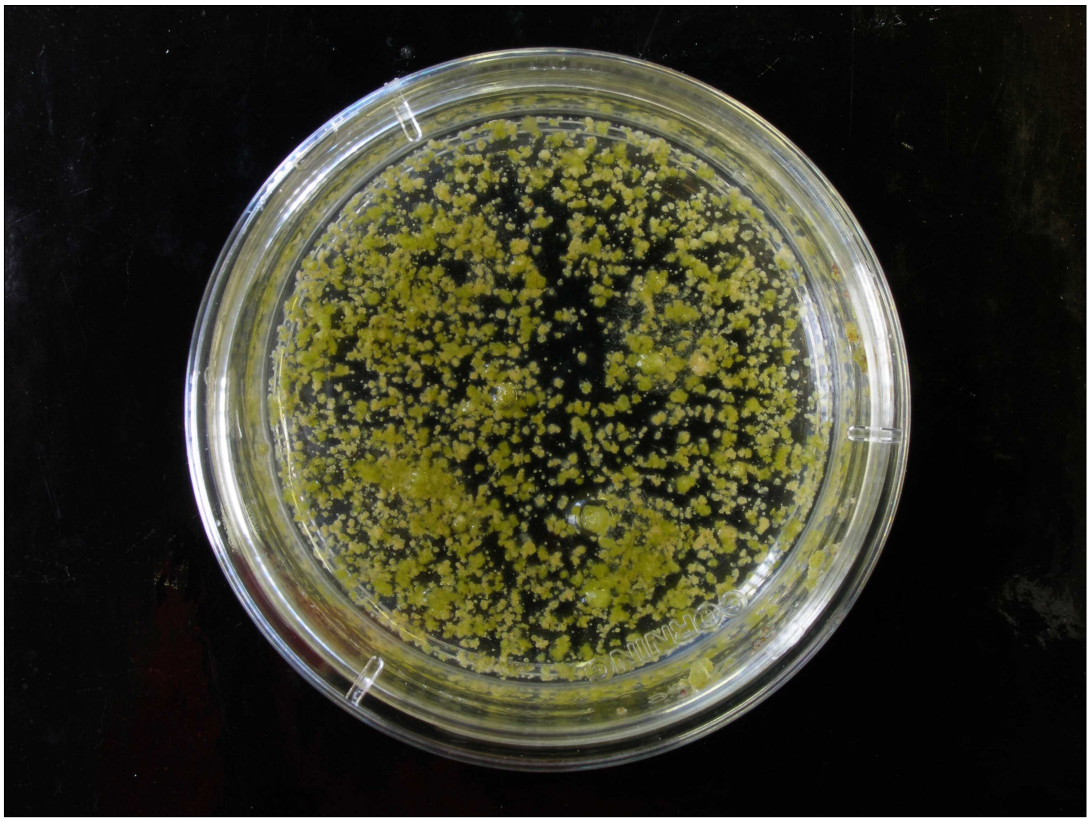

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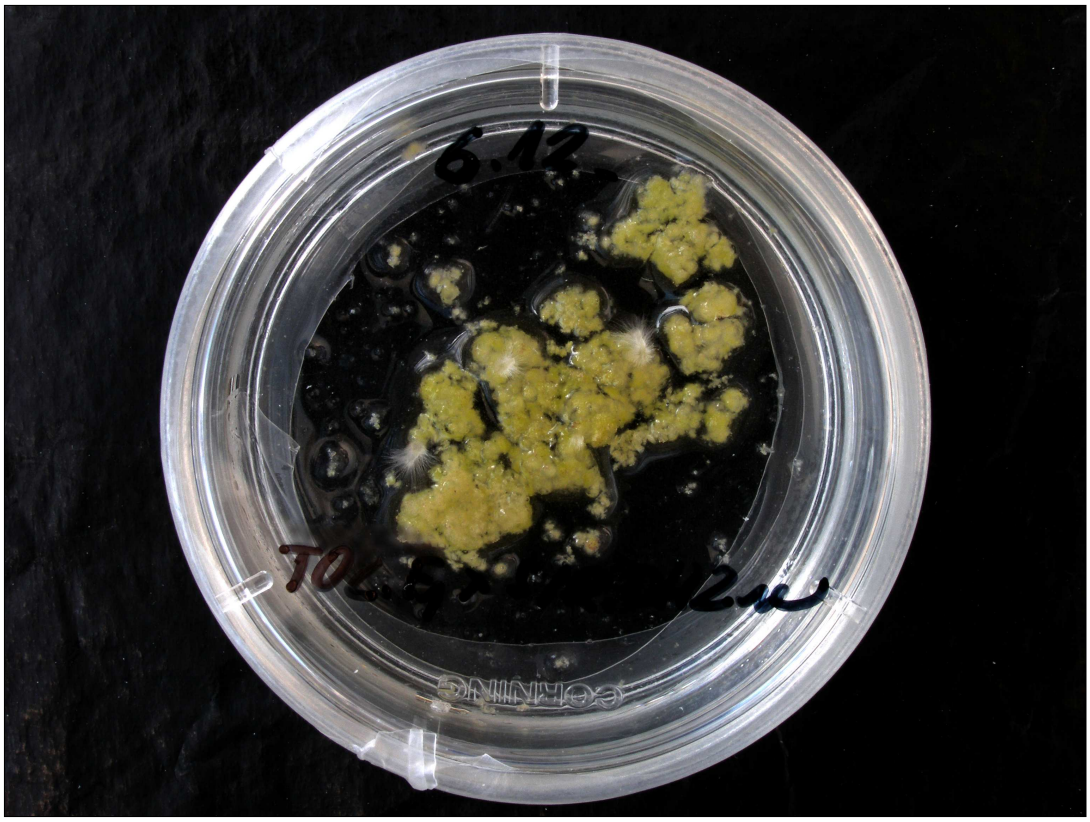

Figure 52